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UNIVERSITY OF ALBERTA

Ionized Calcium and Magnesium Speciation in Complex Solutions

BY

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Hongji Ren

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry

Edmonton, Alberta

Spring, 1995

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To Xiuping Li, Yi Ren, my parents,

and all my teachers

for their love and support

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ABSTRACT

A spectrophotometric method called the Indicator Increment Method (IIM) was developed in which varying amounts of indicator were added to identical samples and the absorbances were measured at two selected wavelengths. Through multiple equilibrium calculations, the free metal ion concentration, as well as the total metal ion concentration and total ligand concentration, in the original system could be estimated. Calmagite was employed as an indicator for free magnesium measurements in solution. The method can be applied only to simple systems with a single ligand present. The type of metal-ligand complex, as well as its approximate conditional stability constant, must be known in advance in order to carry out the calculations.

The second method is based on ion exchange equilibration combined with elemental analysis methods such as flame or inductively coupled plasma / atomic emission spectroscopy. A new calibration procedure was proposed which is suitable for ion exchange resin / sample solution equilibrium under non-trace ion exchange conditions, that is, where the fraction of resin sites occupied by the ion being determined exceeds 1% of the total. Three types of strongly acidic cation exchange resins, Amberlyst 15, Dowex 50W x2 and Dowex 50W x8, were investigated. Dowex 50W x8 was found to be the best for the determination of free calcium and magnesium concentrations in urine samples. Systems containing citrate, phosphate and sulphate were investigated. Experimental free calcium and magnesium values in these solutions were in satisfactory agreement with theoretical values calculated from total metal and total ligand concentrations, pH, and conditional stability constants of the metal-ligand complexes. Zinc and urea did not interfere with the measurements at levels typical for urine samples. Several urine samples as well as a freeze-dried reference urine were analyzed for free calcium and magnesium without difficulty.

The calibration procedure proposed in this thesis makes possible the use of the ion exchange equilibration method under non-trace conditions. It has the advantages that one set of standards is sufficient for calibration for most urine samples even though the pH, ionic strength, and levels of sodium, potassium, calcium and magnesium may vary dramatically. No pretreatment of the urine is needed prior to analysis.

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Chapter 1 Introduction

1.1 *The Importance of Free Metal Ion Determinations*

The activity of a metal ion in solutions containing complexing ligands is directly related to the fraction present in the free, or unbound form, and not to its total concentration. The term 'free ion concentration' will be used in this thesis to refer to the concentration of metal ions coordinated to solvent molecules but not significantly complexed to other ligands in solution. The term 'free' is sometimes replaced with 'non-complexed' or 'ionized' by some authors; we will use the terms interchangeably. Knowledge of the amounts of different species present in solution, especially of free metal ion concentrations, is needed in, among other areas, industrial applications in metallurgy, electroplating, and food processing[1], in clinical measurement of physiologically important ionic calcium and magnesium in serum and urine[2-5], in biomedical investigations of mechanisms such as formation of kidney stones[6-9], and in environmental studies[10-11].

Ideally speciation studies should give the complete composition of a system. For example, a study of a system involving a single metal ion and complexing ligand should answer such questions as (1) what is the total concentration of the metal? (2) what is the total concentration of the ligand? (3) what is the free metal ion concentration and the concentrations of the various forms of metal-ligand complexes? For complicated systems such as biological fluids, which may contain a variety of complexing metals and ligands, it may be difficult to answer all these questions. Because many properties of metal ions are determined by the activities of the solvated (usually hydrated) free ion, however, the determination of their free form concentrations often becomes more important than a complete accounting of all species.

Depending on the metals and ligands present in a given system, the species of interest may be either kinetically inert or labile. Separation techniques such as filtration[12] solvent extraction[13], chromatography[14] and ion exchange[15] can be used for speciation of kinetically inert species, but often cannot be used with labile systems, where care must be taken that the original equilibria are not perturbed[16]. This thesis will focus on the development of methods for free metal ion determinations in kinetically labile systems and will concentrate in particular on non-perturbing methods for the determination of free calcium and magnesium concentrations in urine.

The importance of calcium and magnesium, and of knowledge of their concentrations in biological systems, has been the subject of many books[17-29] and papers[30-36]. Most of these studies worked with total solution concentrations owing to a lack of available methods for measurement of free ion concentrations, even though it has been shown that the ionized, or free, calcium and magnesium forms are the most physiologically active[37-48]. A brief summary of methods reported in the literature for the determination of free metal ions in kinetically labile systems will be given in the next section, with special attention to past work on the specific problem to be studied here, the measurement of free calcium and magnesium concentrations in biological fluids.

1.2 *Recent Developments in Methods for the Determination of Free Metal Ion Concentrations*

In kinetically labile systems free metal ions have been determined electrochemically, using techniques such as ion selective electrode potentiometry and anodic stripping voltammetry, spectrophotometrically with indicators, and by ion exchange equilibration combined with elemental analysis. In this section each of these three methods will be discussed. Other less common methods, including ligand exchange[49-51], size separation-atomic absorption spectroscopy[52-54] and total equilibrium calculations[55-56], will not be discussed further here.

1.2.1 Electrochemical Methods

1.2.1.1 Anodic Stripping Voltammetry

This method involves two steps: a plating step, in which metal ions are reduced to the zero-charged species and form an amalgam with a hanging mercury drop, and a stripping step, in which the metals are oxidized out of the amalgam. Selectivity can be achieved by controlling the potential during the plating step[57]. A major advantage of this method is its high sensitivity; sub-ppb levels of several metals can be determined[58]. A problem with the technique is that surfactants if present in the sample solutions tend to adsorb onto the mercury drop or mercury/metal film and inhibit the electrode reaction, that is, reduction of the metal to form the amalgam[59]. Also, removal of metal ions from solution by the stripping process may cause the original equilibria in the system to be disturbed if the metal ion buffering capacity of the solution is low. Anodic stripping voltammetry has found applications in the speciation of natural water samples, but less commonly in biological fluids[60], likely because of adsorption of organic species.

1.2.1.2 Ion Selective Electrode Potentiometry

An ion selective electrode responds selectively to the activity of an ion in solution without disturbing significantly the equilibria of the original system. It is therefore an ideal approach for free metal ion determinations in kinetically labile systems. Analytical methods using ion selective electrodes have been developed and published in thousands of laboratories throughout the world due to the versatility of the technique (applicable in virtually every laboratory and to a variety of substances), speed (normally a test takes less than a minute) and economy (typically, the cost per test is only a few cents) [61]. The number of metal ions measurable by this method, however, is limited mainly by the lack of selectivity of the electrodes. Commercial metal ion selective electrodes available from one company (Orion) [61] include those for Cd^{2+}, Ca^{2+}, Cu^{2+}, Pb^{2+}, K^{+}, Ag^{+}, Na^{+} and water hardness (M^{2+}). Each is susceptible to a number of interferences, however. Other metal ion selective electrodes have been reported in the literature. Among this group are electrodes for Mg^{2+}[62], Co^{2+}[63], Ba^{2+}[64], Zn^{2+}[65], Ni^{2+}[66], Hg^{2+}[67], Mn^{2+}[68], Li^{+}[69], NH_4^{+}[70], Tl^{+}[71], Au^{3+}[72], Fe^{3+}[73], Cr^{3+} and Al^{3+}[74].

Due to the physiological importance of free ion levels of calcium and magnesium in biological fluids, efforts have been made to develop selective electrodes for these ions. The first design for a calcium electrode, published by Ross in 1967[75], was based on potential measurement across a porous plastic membrane saturated with an organic solvent containing an organophosphate ligand that selectively bound calcium ions. Later, neutral carrier type calcium ligands were developed with properties superior to the organophosphates[44, 76-78]. Magnesium selective electrodes are still in the development stage. The major problem with them is calcium interference[79-80]. In addition, some models show poor selectivity in the presence of sodium and potassium[81], and are sensitive to pH[82-83].

To measure free calcium and magnesium concentrations in biological fluids by means of ion selective electrodes requires the standards to be very similar to the samples. Variations in ionic strength and in junction potentials between standards and samples often limit the accuracy of direct potentiometric measurements. For some applications, however, this is not a major problem. For example, hospitals often perform free calcium measurements on blood, where sample to sample variation of pH and ionic strength is small, and a number of commercial calcium electrodes commonly found today have been developed specifically to eliminate problems associated with measurements in blood. Examples of electrodes designed to measure free calcium ion concentrations in blood include the Orion SS-20[84-85], Orion 98-20[86-87], Orion 99-20[88, 85], Nova-2[89] and Radiometer ICA 1 [90]. Recent work on magnesium electrodes has shown promise for measurements of ionized magnesium in whole blood, plasma, and serum[91-94], and there may be additional applications in the future.

The situation is different for free calcium and magnesium measurements in urine, where wide variations in pH and ionic strength cause difficulties. Although the calcium ion selective electrodes marketed today were not specifically developed for urine samples, several studies have used them to measure free calcium in urine after some precautions[95-102]. One serious problem for some of these electrodes in measuring free calcium in urine is matching of standards to samples in terms of concentrations of sodium, potassium, magnesium and hydrogen ions [96, 100]. Due to the lack of a reference method and reference materials, the accuracy of potentiometric calcium and magnesium measurements in urine for routine clinical work remains unverified[48, 103]. Results often differ, not only between different analyzer systems but also between the same analyzer in different locations, due in part to (1) the dissimilar matrix of the calibration solutions, and (2) variations in the design of the reference electrodes, particularly with regard to the salt-bridge composition and to the dynamics occurring at the liquid-liquid junction[104]. For these reasons, and because there is no electrode available for ionized magnesium measurements in urine, it is clear that a great deal of further work is required on the application of ion selective electrodes to ionized calcium and magnesium measurement in urine.

1.2.2 Spectrophotometric Methods

The principle of the original simple indicator method for the measurement of a free metal ion concentration is to choose an indicator ligand that forms a weak but highly colored or fluorescent complex with the metal ion to be determined. A known amount of the indicator is added to the sample and the concentration of the metal-ligand complex is measured spectrophotometrically. From this concentration, along with knowledge of the total indicator ligand added and the conditional formation constant of the metal-indicator ligand complex, the free metal ion concentration is calculated[105].

The determination of ionized calcium with murexide by Schwarzenbach and Gysling[106] appears to have been the first quantitative study of this kind. Two modifications to the original method have improved and extended its usefulness. The first allows use of indicators of less than 100% purity by absorbance measurements at two wavelengths. This technique has been discussed by Raaflaub[107] and used by several[108-110]. The other modification involves measuring the difference between the absorbance of the solution at two wavelengths[111], an approach that has been used in kinetic studies and in cases where indicator selectivity is poor[112-116].

A number of indicators have been investigated for the spectrophotometric measurement of ionized calcium and magnesium, but few meet the ideal requirements as outlined by Scarpa[114]. Those that have shown some promise include Eriochrome Black T [2, 108] and Erichrome Blue SE [114, 116] for magnesium, and murexide [2, 106, 112, 113, 115] and tetramethylmurexide (TMMA) [4, 5, 110] for calcium. Eriochrome Black T has high selectivity for magnesium over calcium but is sensitive to ionic strength and is affected by the presence of proteins. Also, it is useful only within the pH range of 7.3 to 8, which does not include most physiological values. Eriochrome Blue SE forms complexes with both calcium and magnesium producing differing spectra. The magnesium complex can be measured without calcium interference by choosing two appropriate wavelengths, but other metals may interfere and the system is pH sensitive within the physiological pH range. Therefore, these indicators are not suitable for free magnesium measurement in urine. Murexide, however, has been applied to the analysis of free calcium in urine[117] despite problems of pH dependence and interferences from sodium and protein, as has TMMA, which possesses better properties for this purpose than murexide. TMMA is pH insensitive over the range 4 to 9 [110] and suffers less from interference by protein and sodium. Accordingly it has been used by several to measure free calcium in urine[118-121] and plasma[121-123]. There are still some problems with the TMMA method, however, when applied to urine samples. One is that the color of urine samples may interfere with the spectrophotometric measurement. This drawback can be overcome in some instances by using urine blanks as reference solutions during the measurements. Another problem is that the large variations in sodium ion concentration and in ionic strength that are encountered in urine samples affect the accuracy of the method. Careful matching of standards and samples in terms of sodium concentration and ionic strength is needed, which is inconvenient in practice.

Several limitations apply to the method of direct spectrophotometry for the measurement of free metal ion concentrations. The most severe restriction is that only a small fraction of the labile metal (usually less than 1%) may be allowed to complex with the indicator if the equilibria existing in the original sample are not to be significantly perturbed. This requires that the conditional formation constant of the metal-indicator complex be relatively small and limits the amount of indicator that can be added. This in turn limits the sensitivity of the method. Other problems relate to inadequate selectivity of the indicator, poor stability of the indicator or metal-indicator complex, lack of purity of the indicator, insufficient sensitivity of the metal-indicator complex and the need for matching pH and ionic strength between sample and standards.

Fulton and Kratochvil[105] described a sample ion increment method (SIIM) which removed some of the restrictions of the simple indicator method discussed above. In this method a known amount of indicator ligand is added to a sample and the absorbance of the complex measured. Then a known increment of the ion to be determined is added and the absorbance remeasured. The second measurement determines the degree of sample-ion buffering in the original solution and permits correction for the amount of sample ion complexed by the indicator. With this approach, limitations on the amount of metal-indicator complex formed are removed, thereby reducing previous stringent requirements on the magnitude of the conditional formation constant and molar absorptivity of the metal-indicator complex. This method was applied to the measurement of free calcium in solution with Arsenazo III as indicator[124]. When applied to urine samples, the method requires adjustment of the pH of the samples to 4.6 to bring the conditional formation constant of the calcium-Arsenazo III complex into the proper range, as well as matching of the ionic strength of standards to that of the sample. Because this matching requirement is tedious and slow, and because the need to adjust the pH may alter equilibria in the sample and shift pCa, the method has not seen widespread use.

1.2.3 Ion-Exchange Equilibration Methods

In 1964, Macleod [125] suggested the use of a low-capacity cation membrane for the estimation of ionized calcium and magnesium in serum. Healton [126] applied the method to the determination of ionized magnesium in serum and urine in 1967. The principle of the method is that when strips of cellophane impregnated with congo red dye are placed in serum or urine they adsorb cations in proportion to their concentrations in the sample and their affinities for the dye. The adsorbed ions are eluted by immersion of the membrane in 5% saline and then determined by conventional methods. He found that variations in the levels of sodium, potassium and calcium ions influenced significantly the measurement of magnesium, and concluded that the standard solutions must contain these cations in concentrations similar to those in the specimen under test if accurate results were to be obtained. This matching can be achieved fairly readily with blood serum, whose composition is relatively constant except under extreme conditions, but it is much more difficult with urine where the composition varies widely. Accurate results could be obtained in urine by preliminary measurement of the concentrations of sodium, potassium and calcium in a particular specimen and preparing standards accordingly, but this was not considered practicable.

In 1982 Cantwell, Nielsen and Hrudey [127] proposed a novel ion-exchange column-equilibration method for free metal ion measurements especially suitable for kinetically labile systems. In this method sample solutions are passed through a cation exchange resin column until complete breakthrough of the free metal ion has occurred and equilibrium has been achieved between the resin and the sample solution. After a water wash, the sorbed metal ion is eluted from the resin and measured by atomic absorption spectroscopy.

The theoretical background for the method is as follows. Consider the equilibration of a strong-acid type cation exchange resin with a solution containing a polyvalent metal ion M^{n+} and an electrolyte such as $NaNO_3$. The equilibrium reaction is

$$M^{n+} + n\,RNa \rightleftharpoons R_nM + n\,Na^+ \qquad (1)$$

where R^- represents ion exchange sites in the resin phase.

The corresponding thermodynamic equilibrium constant K is:

$$K = \frac{a_{R_nM} \cdot a^n_{Na^+}}{a_{M^{n+}} \cdot a^n_{RNa}} = \frac{[R_nM][Na^+]^n}{[M^{n+}][RNa]^n} \cdot \frac{\gamma_{R_nM} \cdot \gamma^n_{Na^+}}{\gamma_{M^{n+}} \cdot \gamma^n_{RNa}} \qquad (2)$$

If the univalent ion concentration, represented here by Na^+, is kept constant and large relative to M^{n+} so that the fraction of resin in the R_nM form is less than 1% of the resin capacity, the activity coefficients and [RNa] in Equation (2) become essentially constant. Under these conditions $[M^{n+}]$ is proportional to $[R_nM]$. This is defined as trace ion exchange conditions. Trace conditions are usually obtained by providing a high concentration of univalent cations in the solution phase. For example, in 0.1 M $NaNO_3$ calibration plots for divalent ions are linear up to about 5×10^{-5} M.

There are several advantages to the ion exchange equilibration method. The most important one is that the original equilibria in the sample are only affected by the ionic strength change resulting from addition of an electrolyte such as $NaNO_3$. This makes the method especially suitable for studies of kinetically labile systems. Once equilibrium between the sample and resin has been achieved, the resin phase will take no more metal ion from the solution phase, and effluent from the column has the same composition as the original sample. By keeping the amount of resin in the column small but enough to meet the sensitivity of the detector, metal concentrations as small as 10^{-7} to 10^{-8} M can be measured without long equilibration times. Also, by using a strongly acidic cation exchange resin, the method is insensitive to pH over the physiological range. Furthermore, when a selective technique such as atomic absorption spectroscopy(AAS) or inductively coupled plasma-atomic emission spectroscopy(ICP-AES) is used to analyze the column eluent, many metal ions can be determined without interference.

The major disadvantage of the method is the length of time required for an analysis compared with ion selective electrode potentiometry. Also, trace column conditions are required for reasonable linearity in calibration curves over the concentration range of interest. This is sometimes achieved by adding a quantity of a univalent electrolyte such as $NaNO_3$ to the samples, which may change the original equilibria of the system by altering the ionic strength of the solution, and thereby ion activity coefficients.

The method has been applied to free nickel ion measurements in sewage [127], free copper ion determinations in natural waters [128-129], and ionized calcium and magnesium measurements at micromolar [130] and millimolar levels [131]. An $NaNO_3$ concentration of 0.75 M was needed in the sample solutions to maintain trace column conditions when ionized calcium and magnesium were present at millimolar levels, which is the situation in urine. Clearly the large increase in ionic strength generated by making a urine 0.75 M in electrolyte may shift the original equilibria. Accordingly, an attempt was made to establish conditions whereby free calcium and magnesium concentrations in the millimolar range could be measured under non-trace conditions [16]. To this end a series of standards was prepared containing calcium and magnesium over the concentration range expected in urine samples while keeping the sodium and potassium concentrations constant. The amount of calcium and magnesium in the resin phase was related to the free calcium and magnesium concentrations in the solution phase by a regression calculation. The method was applied to the determination of free calcium and magnesium in a urine standard, NIST SRM2670, whose sodium and potassium concentrations were known. To be successful the method needs standards and samples to be matched in terms of both sodium and potassium concentrations.

1.3 *Research Objectives*

As discussed earlier, knowledge of free calcium and magnesium concentrations in urine may aid in assessing the tendency of some individuals to form calcium-containing kidney stones. The methods currently available for the estimation of free calcium and magnesium concentrations in solutions containing widely varying levels of pH, sodium, potassium, calcium and magnesium are not satisfactory. All of the present methods require matching of standards and samples in one way or another in terms of ionic strength, sodium and potassium concentrations and pH. This creates practical difficulties for their application.

The objective of the present study was to develop new procedures for free metal ion measurements that do not perturb the original sample composition, and to apply them to the determination of free, ionized, calcium and magnesium in urine. Several approaches were investigated. One of these was to develop a variation on the sample ion indicator method mentioned earlier, called the indicator increment method (IIM), for the measurement of free magnesium concentrations in solution. An advantage of the method is that both free and total magnesium ion concentrations can be obtained from a set of spectrophotometric measurements. Calmagite was used as the indicator. Because calmagite spectrophotometric absorbance measurements are affected by both pH and pCa, each of these parameters as well as the ionic strength must be controlled. This study is presented in Chapter 2.

Subsequently a new calibration procedure was developed that allowed the ion exchange equilibration method to be used under non-trace ion exchange conditions. Both flame atomic emission spectroscopy and ICP-AES were used as detectors to measure the quantity of the major metal ions in urine - sodium, potassium, calcium and magnesium - that adsorbed onto the resin phase. Four equilibria between the resin and solution phase were considered: calcium vs. sodium, calcium vs. potassium, magnesium vs. sodium and magnesium vs. potassium. Mixed ion exchange selectivity constants for these four equilibria were defined as combinations of the corresponding thermodynamic constants with activity coefficients in the resin phase. These mixed constants necessarily change from sample to sample, but can be related for a particular column to the fraction of each metal ion in the resin phase, and, for Dowex 50W x2 resin, the ionic strength of the solution phase by calibration against a selected set of standards that contain concentrations of sodium, potassium, calcium and magnesium covering the typical ranges of these ions expected in urine samples. In preparing these standards consideration was given to different combinations of the various metal ions as well as the ammonium ion. The relationship between the mixed resin-solution constants and the amount of each metal ion present in the resin phase was determined by a regression calculation using the experimental data of the standards. These values for the constants were then applied to determine simultaneously the concentrations of free calcium and magnesium at millimolar levels in unknown samples.

An important advantage of the procedure is that one set of standards is sufficient for calibration of a given column. The requirement of matching standards to each individual sample in terms of sodium, potassium, calcium, magnesium and hydrogen ion concentrations has been eliminated. The pH and ionic strength of the urine samples need not be changed because sample pretreatment is not necessary. This allows measurement of free metal ion concentrations in the original unperturbed system. Also, free calcium and magnesium concentrations in urine samples can be measured simultaneously.

Chapter 2 Spectrophotometric Determination of Free, Ionized, Metal Ion Concentrations in Solution by an Indicator Increment Method. Application to the Determination of Free Mg^{2+} with Calmagite

2.1 *Introduction*

Knowing the free or uncomplexed metal ion concentrations in solutions containing bound or complexed metal is often of biological and environmental interest. The free, or hydrated, metal ion is frequently the form that more readily undergoes reaction. The measurement of free ion concentrations has received considerable study and the literature is extensive. The major methods in use at this time are ion selective electrode potentiometry and spectrophotometry, although a number of other techniques have been described, including ion exchange, voltammetry, dialysis, and multiple equilibria calculations from total analysis.

Spectrophotometric methods have found applicability in the determination of metal ions in cells and biological systems. An example is the determination of calcium with Arsenazo III [124]. A modification of the general technique called the Sample Ion Increment Method (SIIM) [105] allows correction for metal tied up by the indicator and gives improved sensitivity over a broader range of sample concentrations. This chapter describes a different approach, called the Indicator Increment Method (IIM), which uses equilibrium calculations based on absorbance measurements following sequential addition of known amounts of indicator. Under some conditions this method allows the estimation of not only the free metal ion concentration but total metal and total ligand concentrations as well.

2.2 *Experimental*

2.2.1 Reagents and Chemicals

A 5 × 10^{-3} M solution of $Mg(NO_3)_2$ was prepared by dissolving 0.6410 g of $Mg(NO_3)_2 \cdot 6H_2O$ (Baker Analyzed Reagent) in 500 mL pure water. Commercial calmagite (Terochem Laboratories, Edmonton, Canada) was analyzed by spectrophotometric titration with a standard solution of $Mg(NO_3)_2$ and was found to have a purity of 69%. This purity was adequate for the two wavelength method used in this study. A 1 M solution of KNO_3 (BDH analytical reagent) was used to adjust the ionic strength of the solutions. It was prepared by dissolving 50.55 g KNO_3 in 500 mL H_2O and passing the resulting solution through a column of Dowex® Chelating Resin (Sigma) to remove polyvalent cations [136]. All other chemicals were analytical grade and used without further purification. All water was distilled, then passed through a Barnstead NANOpure water purification system before use.

2.2.2 Instrumentation

All pH measurements were made with a Fisher Accumet® Model 520 pH/Ion Meter and a glass-calomel electrode pair calibrated with commercial buffers (Fisher). Spectrophotometric measurements were made in a constant temperature room thermostated at 25 ± 1°C on a Hewlett-Packard Model 8451A diode array spectrophotometer with 1-cm quartz cells; the spectra were stored on 3.5 inch floppy disks for later processing.

2.2.3 Procedure

The molar absorptivity values for calmagite at 512 and 616 nm were determined by measuring the absorbance of a series of solutions at pH 10 and ionic strength 0.1. These wavelengths were chosen because the difference between the molar absorptivity values of

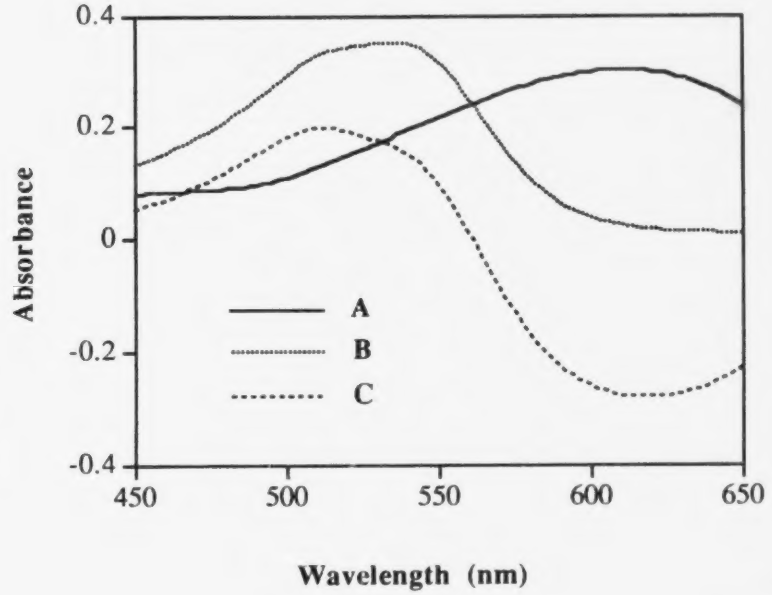


Figure 2.1 Spectra of magnesium-calmagite system. Line A (indicator spectrum): calmagite: 1.55 x 10^{-5} M, KNO_3: 0.1M, pH 10. Water was used as reference. Line B (metal-indicator complex spectrum): Mg: 6 x 10^{-4} M, calmagite: 1.55 x 10^{-5} M, KNO_3: 0.1M, pH 10. Water again used as reference. Line C: Difference spectrum of B and A. The maximum occurs at 512 nm and the minimum at 616 nm.

the Mg^{2+}-calmagite complex and the free indicator was the largest, a desirable condition for the two wavelength method. In Figure 2.1 are shown spectra of the magnesium-calmagite system. All subsequent absorbance measurements were carried out at these two wavelengths. The absorption coefficients for the Mg^{2+}-calmagite complex were determined by measuring, at the wavelengths selected above, a series of solutions containing different excess amounts of Mg^{2+} while the calmagite concentrations were kept constant. Incomplete complexation of calmagite was estimated and corrected for by an iteration method starting with an estimated initial conditional stability constant for the complex. The conditional stability constant K' of the Mg^{2+}-calmagite complex was determined by measuring, at 512 and 616 nm, a series of solution containing different amounts of Mg^{2+} and calmagite. A value of 2.56×10^5 M^{-1} was found under the experimental conditions used here which is comparable with a literature value of 4.87×10^5 M^{-1} reported using continuous variation method in 0.1 M potassium chloride[147].

For the magnesium-nitrilotriacetic acid (NTA) study, increments of 1mM calmagite solution were added to a solution containing Mg^{2+} and NTA. Two additions of indicator provides sufficient information to solve the equations. We usually chose, however, to add indicator solution three times and measure absorbance so that three sets of results could be used in the calculations for each unknown sample solution. The system was in each case brought to an ionic strength μ of 0.1 with 1 M KNO_3 and to a pH of 10 by adjustment with dilute NaOH, followed by addition of a small amount of NH_3-NH_4NO_3 buffer solution. As mentioned above, absorbances were measured at 512 and 616 nm after each addition of increment of calmagite solution.

A similar procedure was followed for the other systems with the exception of the Mg^{2+}-K_2HPO_4 system, where addition of NH_3 buffer solution was not necessary owing to the buffering already provided by the phosphate ligand.

2.3 *Theory*

Equations for the determination of free metal ion concentrations by the Indicator Increment Method(IIM) were developed for three different systems. The first was for a system consisting of a metal ion M and a ligand H_nL that react to form a single metal-ligand complex ML. The second was for the same system but where a series of complexes of the type ML, MHL, MH_2L, ... form, and the third was where the complexes ML and ML_2 both form.

Case 1. Determination of Free Metal Ion Concentration in a Solution Containing Metal Ion, Protonated Ligand, and Metal-Ligand Complex

In the Indicator Increment Method (IIM), the free metal ion concentration $[M]_o$ in an unknown solution is determined by adding a known amount of an indicator H_nI that forms a 1:1 colored complex, $[MI]_i$, with the metal. Charges on the ions have been omitted for simplicity. First $[MI]_i$ is determined, then $[M]_i$, as follows.

The absorbance A_o of a sample solution of volume V_o is measured at the two wavelengths selected. In the experimental system to be used here these are at 512 nm (A_o^{512nm}) and 616 nm (A_o^{616nm}). A known quantity of indicator ligand H_nI is next added and the absorbance A_i again measured (A_i^{512nm} and A_i^{616nm}) at the two wavelengths in the new volume V_i. The difference of the absorbance values at the two wavelengths is used for the calculations ($A = A^{512nm} - A^{616nm}$). Then

$$A_i - A_o\frac{V_o}{V_i} = \varepsilon_{MI}b[MI]_i + \Sigma\varepsilon'\,b\,(F_{I\,i} - [MI]_i) \qquad (1)$$

where

$$\Sigma\varepsilon' = \alpha_{H_nI}\varepsilon_{H_nI} + \alpha_{H_{n-1}I}\,\varepsilon_{H_{n-1}I} + \cdots + \alpha_I\varepsilon_I \qquad (2)$$

The α terms are the fractions of uncomplexed indicator in each of its protonated forms and the ε terms are the corresponding molar absorptivity differences at the two wavelengths of the subscript species. F_{Ii} is the total indicator concentration in the solution being measured. Once $[MI]_i$ has been measured, $[M]_i$ can be obtained from the relation

$$[M]_i = \frac{[MI]_i}{K'_{MI}\cdot\{F_{Ii} - [MI]_i\}} \tag{3}$$

The free metal ion concentration in the initial solution $[M]_o$ may be calculated from the stability constant expression K'_{ML} once $[M_T]_o$ and $[F_L]_o$ are determined. Their values are obtained by use of the expressions described in the next section.

A. Determination of total metal $[M_T]_o$ and total ligand $[F_L]_o$ concentration in a solution containing M, H_nL and ML

Consider a sample solution containing a metal ion M and an auxiliary ligand H_nL for which ML is the only major metal ligand complex formed. The species present under these conditions are M, L, ML, HL, H_2L, ... , H_nL. An indicator H_nI that forms only the single colored complex MI is now added to the sample solution. The total concentration of auxiliary ligand F_L is given by

$$F_L = [\,L\,] + [\,HL\,] + \ldots\ldots + [\,H_nL\,] + [\,ML\,]$$

and the fraction of uncomplexed auxiliary ligand α_L by

$$\alpha_L = \frac{[L]}{F_L - [ML]}$$

The conditional stability constant K'_{ML} for formation of ML is the product of the thermodynamic stability constant K_{ML} and α_L:

$$K'_{ML} = K_{ML}\,\alpha_L = \frac{[ML]}{[M]\{F_L - [ML]\}}$$

Rearranging gives

$$[ML] = \frac{K'_{ML}\,F_L\,[M]}{1 + K'_{ML}\,[M]} \tag{4}$$

If now a solution containing indicator H_nI is added to an original sample solution having a total metal ion concentration of $[M_T]_o$ and a total ligand concentration of $[F_L]_o$, both complexation of metal by the indicator and dilution occurs. Under these conditions

$$[F_L]_i = [F_L]_o\,V_o/V_i \quad \text{and} \quad [M_T]_i = [M_T]_o\,V_o/V_i = [M]_i + [ML]_i + [MI]_i$$

Then

$$[ML]_i = [M_T]_o\,V_o/V_i - [M]_i - [MI]_i \tag{5}$$

From equation (4), $[ML]_i$ can also be written as

$$[ML]_i = \frac{V_o}{V_i}\,\frac{K'_{ML}\,[F_L]_o\,[M]_i}{1 + K'_{ML}\,[M]_i} \tag{6}$$

Combining equations (5) and (6) and rearranging,

$$[M_T]_o V_o/V_i + V_o/V_i [M]_i K'_{ML}\{[M_T]_o - [F_L]_o\}$$
$$- \{[M]_i([M]_i + [MI]_i)K'_{ML} + ([M]_i + [MI]_i)\} = 0 \tag{7}$$

Separating terms in $[M_T]_o$ and $[F_L]_o$,

$$(V_o/V_i + [M]_i K'_{ML} V_o/V_i) [M_T]_o - ([M]_i K'_{ML} V_o/V_i) [F_L]_o$$
$$- ([M]_i + [MI]_i) (1 + K'_{ML}[M]_i) = 0 \tag{8}$$

If $[MI]_i$, $[M]_i$, V_o, V_i and K'_{ML} are known, equation (8) becomes an expression of the form

$$a_i [M_T]_o + b_i [F_L]_o + c_i = 0 \tag{9}$$

where

$$a_i = V_o/V_i + [M]_i K'_{ML} V_o/V_i$$
$$b_i = - [M]_i K'_{ML} V_o/V_i$$

and

$$c_i = - ([M]_i + [MI]_i)(1 + K'_{ML}[M]_i)$$

If indicator solution is added twice and absorbance measurements are made after each addition, two equations of the form of equation (9) can be written, from which values of $[M_T]_o$ and $[F_L]_o$ can be obtained.

B. Calculation of $[M]_o$

The value of $[M]_o$ may now be obtained from the K'_{ML} expression

$$K'_{ML} = \frac{[ML]_o}{[M]_o ([F_L]_o - [ML]_o)} = \frac{[M_T]_o - [M]_o}{[M]_o([F_L]_o - [M_T]_o + [M]_o)} \tag{10}$$

by rearrangement to quadratic form,

$$K'_{ML}[M]_o^2 + \{K'_{ML}([F_L]_o - [M_T]_o) + 1\}[M]_o - [M_T]_o = 0 \qquad (11)$$

Solution of this equation gives the free metal ion concentration in the original system $[M]_o$.

Case 2. Determination of $[M_T]_o$, $[F_L]_o$ and $[M]_o$ When Protonated Metal-Complexes of the Type ML, MHL, MH_2L, ... Form

If a series of soluble metal complexes form that contain protons or hydroxyl ions, and the conditional overall stability constants are known for each of them, then a modification of the previously described approach can be used. For the simplest case, when the only additional species is MHL, the mass balance equations for the original solution are

$$[M_T]_o = [M]_o + [ML]_o + [MHL]_o \qquad (12)$$

and

$$[F_L]_o = [L]_o + [HL]_o + [ML]_o + [MHL]_o \qquad (13)$$

For ML in this system,

$$K'_{ML} = K_{ML}\alpha_L = \frac{[ML]_o}{[M]_o\{[F_L]_o - ([ML]_o + [MHL]_o)\}} \qquad (14)$$

while for MHL

$$K'_{MHL} = K_{MHL}\alpha_{HL} = \frac{[MHL]_o}{[M]_o\{[F_L]_o - ([ML]_o + [MHL]_o)\}} \qquad (15)$$

Combining equations (14) and (15) gives

$$[ML]_o + [MHL]_o = \frac{[M]_o[F_L]_o\,(K'_{ML} + K'_{MHL})}{1 + [M]_o\,(K'_{ML} + K'_{MHL})} \qquad (16)$$

For the more general case where polyprotonated metal ligand complexes of the type MH_2L or MH_3L form in significant concentrations, equation (16) becomes

$$[ML]_o + [MHL]_o + [MH_2L]_o \cdots = \frac{[M]_o[F_L]_o\,(K'_{ML} + K'_{MHL} + K'_{MH_2L} + \cdots)}{1 + [M]_o\,(K'_{ML} + K'_{MHL} + K'_{MH_2L} + \cdots)}$$

$$= \frac{[M]_o[F_L]_o\,K'_{\Sigma}}{1 + [M]_o\,K'_{\Sigma}} \qquad (17)$$

A similar expression can be written to take into account the formation of complexes containing hydroxyl ions of the type $M(OH)_xL$.

When indicator is added as described in case 1, solution conditions are shifted from "o" to "i", giving

$$[F_L]_i = V_o/V_i[F_L]_o \quad \text{and} \quad [M_T]_i = V_o/V_i\ [M_T]_o$$

From equation (17)

$$[ML]_i + [MHL]_i + [MH_2L]_i \cdots = \frac{V_o}{V_i}\,\frac{[M]_i[F_L]_o\,K'_{\Sigma}}{1 + [M]_i\,K'_{\Sigma}} \qquad (18)$$

and from mass balance

$$[ML]_i + [MHL]_i + [MH_2L]_i \cdots = \frac{V_o}{V_i}\,[M_T]_o - [M]_i - [MI]_i \qquad (19)$$

Combining equations (18) and (19), and rearranging, gives

$$\{\frac{V_o}{V_i}(1 + [M]_i K'_\Sigma)\}[M_T]_o - \{\frac{V_o}{V_i}[M]_i K'_\Sigma\}[F_L]_o - \{([M]_i^2 + [M]_i [MI]_i)K'_\Sigma + [M]_i + [MI]_i\} = 0 \qquad (20)$$

This is an equation of the form

$$a_1[M_T]_o + b_1[F_L]_o + c_1 = 0 \qquad (21)$$

Addition of the second increment of indicator and remeasurement gives a second equation

$$a_2 [M_T]_o + b_2 [F_L]_o + c_2 = 0 \qquad (22)$$

which when solved simultaneously with equation (21) gives values for $[M_T]_o$ and $[F_L]_o$. A value for $[M]_o$ can then be obtained from equations (12) and (16).

Case 3. Estimation of $[M_T]_o$, $[F_L]_o$ and $[M]_o$ when Metal-Ligand Complexes ML and ML_2 Form

When both 1:1 and 1:2 metal-ligand complexes form, the relationships are:

$$[M_T]_o = [M]_o + [ML]_o + [ML_2]_o$$

and

$$[F_L]_o = [L]_o + [HL]_o + [H_2L]_o + \ldots + [H_nL]_o + [ML]_o + 2[ML_2]_o$$

let

$$[F_L]_o^* = [L]_o + [HL]_o + [H_2L]_o + \ldots + [H_nL]_o$$

Then

$$[F_L]_o = [F_L]_o^* + [ML]_o + 2[ML_2]_o \tag{23}$$

Since

$$\beta'_{ML} = \frac{[ML]_o}{[M]_o[F_L]_o^*} \qquad \text{and} \qquad \beta'_{ML_2} = \frac{[ML_2]_o}{[M]_o[F_L]_o^{*\,2}}$$

then

$$[ML]_o + [ML_2]_o = \beta'_{ML}[M]_o[F_L]_o^* + \beta'_{ML_2}[M]_o[F_L]_o^{*\,2} \tag{24}$$

On addition of indicator H_nI,

$$[ML]_i + [ML_2]_i = \beta'_{ML}[M]_i[F_L]_i^* + \beta'_{ML_2}[M]_i[F_L]_i^{*\,2} \tag{25}$$

and, from mass balance for $[M_T]$,

$$[ML]_i + [ML_2]_i = \frac{V_o}{V_i}[M_T]_o - [M]_i - [MI]_i \tag{26}$$

When $[F_L]_o >> [M_T]_o$, then $[F_L]_o^* \approx [F_L]_o$, and $[F_L]_i^* \approx [F_L]_o^*(V_o/V_i)$

Substituting into eq. [25] and combining with [26], gives on rearrangement

$$\frac{V_o}{V_i}[M_T]_o - \frac{V_o}{V_i}\beta'_{ML}[M]_i[F_L]_o^* - \left(\frac{V_o}{V_i}\right)^2\beta'_{ML_2}[M]_i[F_L]_o^{*\,2} - ([M]_i + [MI]_i) = 0 \tag{27}$$

Upon addition of two increments of indicator solution and measurement of [MI] after each addition, values of V_o, V_1, V_2, $[M]_1$, $[M]_2$, $[MI]_1$, and $[MI]_2$ can be obtained. With knowledge of β'_{ML} and β'_{ML_2}, the pairs of equations

$$a_1[M_T]_o + b_1[F_L]_o^* + c_1[F_L]_o^{*2} + d_1 = 0$$

and

$$a_2[M_T]_o + b_2[F_L]_o^* + c_2[F_L]_o^{*2} + d_2 = 0$$

can be solved to obtain $[M_T]_o$ and $[F_L]_o^*$. Then $[M]_o$ can be found from

$$[M]_o = \frac{[M_T]_o}{1 + \beta'_{ML} \cdot [F_L]_o^* + \beta'_{ML_2} \cdot [F_L]_o^{*\,2}} \tag{28}$$

2.4 *Results and Discussion*

2.4.1 Application of Case 1 to the Mg^{2+}-Nitrilotriacetic Acid System

The ligand NTA (nitrilotriacetic acid), which is reported to form only a 1:1 complex with magnesium[141], was selected to test the applicability of Case 1. The conditional stability constant of the Mg^{2+}-NTA complex in concentration terms (log K'= 5.31) was obtained from the literature[141] by correction with a partition factor of the ligand (L^{-3}) at pH 10 (α_L-3 = 0.69 at this pH).

An additional factor that was considered was possible formation of the hydroxyl ion complex of magnesium at pH 10. The stability constant of $MgOH^+$ at ionic strength 0.1 has not been reported in the literature, but a value of 2.1 for log K_{MgOH} was calculated from the value at infinite dilution, 2.58 [139] and the extended Debye-Huckel equation. Using this value, the fraction of magnesium present as $MgOH^+$ at pH 10 was estimated to be 0.012, a level sufficiently small to be neglected for the purpose of this work. Another possible competing ligand was NH_3 coming from the small amount of NH_3 buffer used. The final concentration of NH_3 in the solution, 3×10^{-3}M, is so small, however, that the formation of Mg^{2+}-NH_3 can be considered negligible.

Measurements were made using the procedure described above on initial sample volumes of 20 mL. To each set of Mg^{2+}-NTA sample solutions three increments of indicator were added (0.4, 0.6, and 0.8 mL of 1.06×10^{-3} M Calmagite). The addition of three increments of indicator allowed the construction of three pairs of equations (1,2; 1,3; 2,3) which could be used to solve for $[M_T]_o$, $[F_L]_o$, and, ultimately, $[M]_o$. After each addition of indicator, the absorbance of the resulting solution was measured three times to provide an estimate of measurement error. In this way 27 values were obtained for each set of original Mg^{2+}-NTA solutions, as shown in Tables 1a to 1c. In each case the final volume after addition of indicator solution was brought to 25 mL with pure water.

An important part of the strategy involved in applying the IIM method is selection of the proper amount of indicator to be added as each successive increment. The amount added should be sufficient to cause a significant equilibrium shift in the original system, since the larger the shift the more accurate the results. It should not be too large, however, or the initial ionic strength of the solution will be shifted; this will in turn affect the conditional stability constants of both the metal-indicator and the metal-auxiliary ligand complexes.

Calculations were carried out on a Macintosh computer using a BASIC program written for the purpose. The program is listed in Appendix I. A summary of the calculations is shown in Tables 2.1a to 2.1c. It can be seen from the tables that the agreement between the experimental and theoretical calculated values are acceptable considering the low concentrations measured.

Table 2.1a Determination of total magnesium, total NTA, and ionic magnesium in sample solution 1 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_{o}		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	4.34	4.37	4.39	3.93	3.96	3.99	5.56	5.55	5.54
	4.33	4.36	4.38	3.91	3.95	3.98	5.57	5.55	5.54
	4.31	4.36	4.37	3.89	3.95	3.97	5.57	5.56	5.55
	4.36	4.37	4.40	3.95	3.97	4.01	5.55	5.55	5.53
	4.34	4.37	4.39	3.92	3.96	4.00	5.56	5.55	5.53
	4.33	4.36	4.39	3.90	3.95	3.99	5.56	5.55	5.54
	4.37	4.38	4.42	3.96	3.97	4.03	5.55	5.55	5.52
	4.35	4.37	4.41	3.94	3.97	4.02	5.56	5.55	5.52
	4.34	4.37	4.40	3.92	3.96	4.01	5.56	5.55	5.53
Average, SD	4.37 ± 0.03			3.96 ± 0.04			5.55 ± 0.01		
Added	4.30			4.00			5.38 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total NTA concentrations

Table 2.1b Determination of total magnesium, total NTA, and ionic magnesium in sample solution 2 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_o		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	4.46	4.33	4.14	3.96	3.79	3.55	5.69	5.72	5.78
	4.45	4.31	4.09	3.95	3.77	3.49	5.69	5.73	5.79
	4.44	4.30	4.05	3.93	3.76	3.45	5.69	5.73	5.80
	4.47	4.34	4.15	3.98	3.80	3.57	5.68	5.72	5.78
	4.47	4.32	4.10	3.97	3.78	3.51	5.68	5.72	5.79
	4.46	4.31	4.07	3.95	3.77	3.47	5.69	5.72	5.79
	4.49	4.35	4.18	4.01	3.82	3.59	5.67	5.71	5.77
	4.49	4.34	4.13	4.00	3.80	3.54	5.67	5.72	5.78
	4.48	4.33	4.09	3.98	3.79	3.50	5.68	5.72	5.79
Average, SD	4.30 ± 0.15			3.76 ± 0.19			5.73 ± 0.04		
Added	4.30			3.90			5.53 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total NTA concentrations

Table 2.1c Determination of total magnesium, total NTA, and ionic magnesium in sample solution 3 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_{o}		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	4.33	4.32	4.32	3.69	3.69	3.68	5.85	5.85	5.85
	4.31	4.29	4.26	3.67	3.65	3.61	5.85	5.85	5.86
	4.26	4.27	4.21	3.62	3.63	3.55	5.86	5.86	5.87
	4.36	4.34	4.33	3.73	3.71	3.70	5.84	5.84	5.85
	4.35	4.31	4.27	3.72	3.68	3.63	5.84	5.85	5.86
	4.30	4.29	4.23	3.66	3.65	3.58	5.85	5.85	5.87
	4.39	4.36	4.37	3.77	3.73	3.75	5.83	5.84	5.83
	4.38	4.33	4.32	3.76	3.70	3.69	5.83	5.84	5.85
	4.34	4.31	4.28	3.71	3.68	3.64	5.84	5.85	5.85
Average, SD	4.31 ± 0.04			3.68 ± 0.05			5.85 ± 0.01		
Added	4.30			3.82			5.64 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total NTA concentrations

2.4.2 Application of Case 2 to the Magnesium-K_2HPO_4 System

The ligand K_2HPO_4 was selected as a test system to verify the conditions described in Case 2 because it forms magnesium complexes mainly in the form of $MgPO_4^-$ and $MgHPO_4$. At pH 10 phosphate exists mainly in the form of HPO_4^{2-}(98.2%) with the remainder PO_4^{3-}(1.8%). The conditional stability constant for the $MgPO_4^-$ and $MgHPO_4$ species in concentration terms was calculated from literature values by correction to pH 10. Also potassium ion complexation was corrected for by a simple equation, $K_{MgL} = K'_{MgL}(1+K_{KL}[K^+])$. Ionic strength corrections were made with activity coefficients calculated using the Davies equation in the form of

$$\log(\gamma_i) = -AZ_i^2\left\{\frac{\sqrt{\mu}}{1-\sqrt{\mu}} - 0.3\mu\right\} \qquad (29)$$

where γ_i is the activity coefficient of ionic species i, A is a constant equal to 0.51 for aqueous solutions at 25°C, Z_i is the charge on species i, and μ is the ionic strength of the solution. A log K'_Σ (here $K'_\Sigma = K'_{MgPO_4^-} + K'_{MgHPO_4}$) value of 2.23 was obtained at ionic strength 0.1 and pH 10. A total ligand concentration of 0.02 M was chosen for this study. Under these conditions the ligand was found to serve as a satisfactory buffer once the solution was adjusted to pH 10 with dilute NaOH, thereby eliminating the need for an independent buffer. The wavelengths used as well as the other conditions (number of measurements, indicator amounts, computer calculations, etc.) were all as in Case 1. Results are shown in Tables 2.2a to 2.2c. From these tables, we can see that the results for $[M]_o$ agree quite well with the theoretical values. Values for $[M_T]_o$ and $[F_L]_o$ also agree well with those added initially.

Table 2.2a Determination of total magnesium, total K_2HPO_4, and ionic magnesium in solution 1 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_o		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	5.09	5.07	5.05	1.91	1.85	1.75	5.58	5.60	5.65
	5.07	5.06	5.03	1.87	1.83	1.70	5.59	5.61	5.67
	5.06	5.05	5.01	1.82	1.79	1.63	5.61	5.62	5.70
	5.10	5.07	5.06	1.96	1.87	1.82	5.55	5.59	5.62
	5.08	5.07	5.05	1.91	1.85	1.77	5.57	5.60	5.64
	5.07	5.05	5.02	1.86	1.82	1.69	5.59	5.61	5.68
	5.11	5.08	5.08	2.01	1.90	1.89	5.53	5.57	5.58
	5.09	5.07	5.06	1.96	1.88	1.84	5.55	5.58	5.60
	5.08	5.06	5.04	1.91	1.85	1.76	5.57	5.60	5.64
Average, SD	5.06 ± 0.02			1.83 ± 0.09			5.60 ± 0.04		
Added	4.90			1.60			5.63 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total phosphate concentrations

Table 2.2b Determination of total magnesium, total K_2HPO_4, and ionic magnesium in solution 2 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_{o}		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	4.69	4.68	4.68	1.66	1.64	1.62	5.37	5.37	5.38
	4.68	4.68	4.67	1.64	1.64	1.60	5.37	5.37	5.39
	4.67	4.68	4.67	1.63	1.63	1.60	5.38	5.38	5.39
	4.70	4.69	4.68	1.70	1.66	1.64	5.35	5.36	5.37
	4.70	4.69	4.68	1.68	1.66	1.63	5.36	5.37	5.38
	4.69	4.68	4.67	1.67	1.65	1.62	5.36	5.37	5.38
	4.70	4.69	4.69	1.70	1.66	1.66	5.35	5.36	5.37
	4.70	4.69	4.68	1.68	1.66	1.64	5.36	5.37	5.37
	4.69	4.68	4.68	1.67	1.65	1.63	5.36	5.37	5.38
Average, SD	4.68 ± 0.01			1.65 ± 0.02			5.37 ± 0.01		
Added	4.60			1.60			5.35 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total phosphate concentrations

Table 2.2c Determination of total magnesium, total K_2HPO_4, and ionic magnesium in solution 3 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_{o}		
	1,2	1,3	2,3*	1,2	1,3	2,3*	1,2	1,3	2,3*
Found	4.39	4.39	4.39	1.67	1.67	1.66	5.06	5.06	5.06
	4.39	4.39	4.39	1.66	1.66	1.66	5.06	5.06	5.06
	4.39	4.39	4.38	1.66	1.66	1.64	5.06	5.06	5.07
	4.38	4.38	4.39	1.64	1.65	1.67	5.07	5.07	5.06
	4.37	4.38	4.39	1.63	1.65	1.67	5.07	5.07	5.06
	4.37	4.38	4.39	1.63	1.64	1.66	5.07	5.07	5.06
	4.39	4.39	4.39	1.67	1.66	1.67	5.06	5.06	5.06
	4.38	4.39	4.39	1.65	1.66	1.67	5.06	5.06	5.06
	4.39	4.38	4.38	1.66	1.66	1.66	5.06	5.06	5.06
Average, SD	4.39 ± 0.01			1.66 ± 0.01			5.06 ± 0.01		
Added	4.30			1.60			5.02 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total phosphate concentrations

2.4.3 Application of Case 3 to the Magnesium-Oxalic Acid System

Oxalate, $C_2O_4^{2-}$, forms two complexes with magnesium, ML and ML_2. Formation of ML_3 is negligible at pH 10. This ligand, therefore, was a convenient choice to evaluate the approach outlined in Case 3. The conditional stability constants were calculated from the literature values (575 and 17400 for K_{ML} and K_{ML2} [138]) at pH 10. A small amount of NH_3 buffer (final $C_{NH3} = 3 \times 10^{-3}$ M) to control the pH was added to the system after initial adjustment to near pH 10 with NaOH. All other conditions were the same as in Case 1. From the results shown in Tables 2.3a to 2.3c, it can be seen that agreement is similar to that found in the two previous cases and is quite satisfactory.

2.5 *Conclusions*

A new method of measuring free metal ion concentrations, called the indicator increment method, has been developed. The approach was applied to the determination of magnesium with three different ligand systems, NTA, PO_4^{3-}, and $C_2O_4^{2-}$, using calmagite as the indicator. These systems represented the three types referred to in Section 2.3, that is $M + H_nL$ form ML, a series of the type ML, MHL, MH_2L ...., or ML and ML_2. Other types of systems such as ML_3 or M_2L connot be handled by the method as outlined. An advantage of the method is that both total metal ion and total ligand concentrations can be obtained in addition to the concentration of free metal ion. A disadvantage of the method is that preliminary information on the ligand and its conditional stability constant with the metal must be available or established beforehand. Also, use of the magnesium-calmagite indicator system requires measurements to be made at a known pH near 10 and at known ionic strength. Other metals that form complexes with calmagite, such as calcium, may interfere by competing with magnesium for the indicator. However, the approach is well

Table 2.3a Determination of total magnesium, total $H_2C_2O_4$, and ionic magnesium in solution 1 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_{o}		
	1,2	1,3	2,3 *	1,2	1,3	2,3 *	1,2	1,3	2,3 *
Found	4.64	4.60	4.55	2.13	2.06	1.98	5.44	5.46	5.51
	4.64	4.59	4.54	2.12	2.05	1.96	5.44	5.46	5.51
	4.63	4.59	4.53	2.11	2.05	1.95	5.44	5.47	5.52
	4.65	4.60	4.56	2.14	2.07	1.99	5.43	5.46	5.50
	4.64	4.60	4.55	2.13	2.06	1.97	5.44	5.46	5.51
	4.64	4.59	4.54	2.12	2.05	1.96	5.44	5.46	5.51
	4.65	4.60	4.57	2.13	2.07	2.00	5.43	5.46	5.49
	4.64	4.60	4.55	2.12	2.06	1.98	5.44	5.46	5.50
	4.63	4.59	4.55	2.11	2.05	1.97	5.44	5.46	5.51
Average, SD	4.59 ± 0.04			2.05 ± 0.06			5.47 ± 0.03		
Added	4.60			2.00			5.53 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total oxalate concentrations

Table 2.3b Determination of total magnesium, total $H_2C_2O_4$, and ionic magnesium in solution 2 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			$pM_{\,o}$		
	1,2	1,3	2,3 *	1,2	1,3	2,3 *	1,2	1,3	2,3 *
Found	4.28	4.25	4.23	2.03	2.00	1.97	5.17	5.18	5.19
	4.27	4.25	4.22	2.02	2.00	1.96	5.17	5.18	5.20
	4.27	4.25	4.22	2.03	1.99	1.96	5.17	5.18	5.20
	4.26	4.24	4.24	2.00	1.99	1.98	5.18	5.18	5.19
	4.25	4.24	4.23	1.99	1.98	1.97	5.18	5.19	5.19
	4.25	4.24	4.23	2.00	1.98	1.96	5.18	5.19	5.19
	4.26	4.25	4.24	2.01	1.99	1.97	5.18	5.18	5.19
	4.26	4.24	4.23	2.00	1.99	1.96	5.18	5.18	5.20
	4.26	4.24	4.22	2.01	1.98	1.96	5.18	5.19	5.20
Average, SD	4.25 ± 0.02			1.99 ± 0.02			5.18 ± 0.01		
Added	4.30			2.00			5.23 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total oxalate concentrations

Table 2.3c Determination of total magnesium, total $H_2C_2O_4$, and ionic magnesium in solution 3 by the indicator increment method.

	$pM_{T\,o}$			$pF_{L\,o}$			pM_o		
	1,2	1,3	2,3 *	1,2	1,3	2,3 *	1,2	1,3	2,3 *
Found	4.20	4.13	4.04	2.15	2.05	1.93	4.97	5.00	5.04
	4.21	4.13	4.05	2.15	2.05	1.94	4.97	5.00	5.04
	4.21	4.13	4.06	2.16	2.06	1.95	4.97	5.00	5.04
	4.19	4.12	4.04	2.13	2.04	1.92	4.98	5.00	5.05
	4.19	4.12	4.04	2.13	2.04	1.93	4.98	5.00	5.04
	4.20	4.13	4.05	2.14	2.05	1.94	4.98	5.00	5.04
	4.18	4.12	4.03	2.12	2.03	1.92	4.98	5.01	5.05
	4.19	4.12	4.04	2.12	2.04	1.93	4.98	5.01	5.05
	4.19	4.12	4.05	2.13	2.04	1.94	4.98	5.00	5.04
Average, SD	4.12 ± 0.06			2.04 ± 0.08			5.01 ± 0.03		
Added	4.12			2.00			5.02 **		

* Combinations of increments used in calculation

** Theoretical value for free magnesium calculated from initial total metal and total oxalate concentrations

suited to situations where species concentrations in a defined metal-ligand system need to be monitored.

It should be noted that the conditional stability constant for the metal-ligand complex need not be known precisely. In the Mg-NTA case, for example, use of a value 10,000 times smaller affected the results for $[M]_0$ by less than 10%. The predicted total ligand concentration, on the other hand, was greatly increased. Like the SIIM method [105], the IIM method established here is useful for metal-ligand systems with low metal ion buffer capacity, that is, the free metal ion concentration will be reduced significantly after indicator addition, a situation where the conventional spectrophotometric method is not applicable.

Chapter 3 Determination of Free Calcium and Magnesium in Urine Samples by an Ion-Exchange Equilibrium - ICP Method

3.1 *Introduction*

Knowledge of a free metal ion concentration is often useful in biological and clinical studies. A spectrophotometric method, called the indicator increment method (IIM), was described in the previous chapter for the measurement of free magnesium in solution. The method was only applicable to simple systems where the properties of the complexing ligand were known, and was not suitable for complicated solutions such as urine. In this chapter, a method using ion-exchange equilibration is investigated for the determination of free metal ion concentrations in solution. The objective is to develop a method suitable for the estimation of free calcium and magnesium in urine. Earlier work in this group has shown that there are two ways of doing this with the ion-exchange equilibration method. One way is to measure the concentration of sodium and potassium in the urine and add additional amounts of these two ions until trace ion exchange conditions are achieved. This procedure has two drawbacks. One is that it is inconvenient because it requires a preliminary determination of the sodium and potassium. The second is that it may be inaccurate if original equilibria are affected by shifts in ionic strength. Another way of applying the ion-exchange equilibrium method to urine is to carry out the measurement under non-trace conditions and prepare calibration plots from standards which match the samples in terms of both sodium and potassium concentrations. Both of these methods are inconvenient for routine use.

In this chapter a new calibration method is described which allows measurements of urine under non-trace ion exchange conditions without the need to match standards and samples in terms of sodium and potassium concentrations. That is, a single set of properly designed standards is sufficient for a wide range of urine samples, and pretreatment or modification of the urine is not necessary. This prevents the possibility of shifts in the original equilibrium system.

3.2 *Experimental*

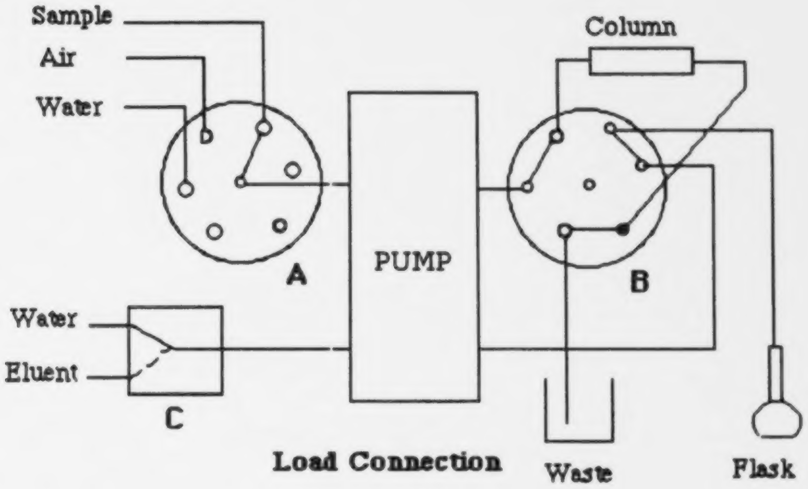
3.2.1 Flow System

The flow system (Figure 3.1) was adapted from an earlier system [16], with the modification that the eluent from the column was collected in a volumetric flask instead of being fed directly to an atomic absorption spectrophotometer.

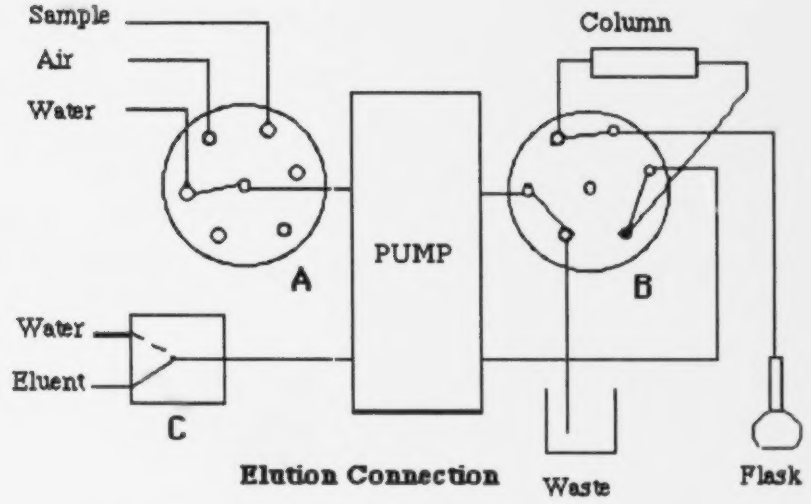
3.2.2 Column Construction

The ion-exchange resin column used in this study was prepared by the method described in reference [16]. The resin is Amberlyst 15 (Rohm and Haas Co., Philadelphia, USA) strong cation exchanger with particles in the size range of 200 to 325 mesh. The original material, which had a listed particle range of 120 to 325 mesh, was sieved and the fines removed using the following procedure.

Sieve 2.0 g of resin through a 200-mesh sieve, using a mortar and pestle to break up the larger particles, then through a 325-mesh sieve to remove most of the fine particles. Some of the very fine particles cling to the larger ones and are not removed by sieving. To remove these fines put the 200 to 325-mesh fraction of the resin in a 50-mL graduated cylinder, add 40 mL water, stir, and wait about 10 minutes until the bulk of the resin settles, leaving any superfine particles in suspension in the supernatant. Decant the supernatant


Sample
Air
Water
A
PUMP
Column
B
Water
Eluent
C
Waste
Flask

Load Connection


Sample
Air
Water
A
PUMP
Column
B
Water
Eluent
C
Waste
Flask

Elution Connection

Figure 3.1 Flow system for ion-exchange equilibration method described in this chapter. A represents a six-port rotary valve, B a rotary sample injection valve, and C a three-way slider valve. The upper figure shows the valve positions during column loading and washing; the lower figure shows the positions during column elution.

and discard. Repeat this washing cycle several times (four was sufficient in this study) until the supernatant is no longer cloudy. Place the de-fined resin, which now has a particle size of 45 to 75 μm, in a 10 to 20-μm sintered glass funnel and wash with the following sequence of reagents, rinsing with pure water at the beginning and between each reagent wash: 0.1M NaOH, 1M HNO_3, 0.01M Na-EDTA, 0.1M NaOH, 1M HNO_3, and CH_3OH. After a final wash with water allow the resin to dry at room temperature.

The column to contain the resin was constructed of polyethylene tubing with glass frits in each end by the method described in reference[16]. The length of the resin bed in the column was about 4 cm and contained 25 mg of resin. The total length of the column was about 7 cm, with an inner diameter of 0.15 cm.

3.2.3 Chemicals

Stock solutions of 0.1 M $Ca(NO_3)_2$, 0.1 M $Mg(NO_3)_2$, 0.5 M $NaNO_3$, 0.5 M KNO_3, and 2 M HNO_3 were prepared from reagent grade chemicals and distilled water that had been treated with a Barnstead Nanopure water purification unit. A 1 M $LiNO_3$ solution was prepared by dissolving 17.4 g of $LiNO_3$ (BDH, lot 0671620) in 200 mL of pure H_2O.

3.3 *Instrumental*

The concentrations of Ca and Mg in the column eluent were determined using a Leco Plasmarray ICP Spectrometer under the following conditions:

1) ICP Parameters:

Forward Power:	1.9	kw
Reflected Power:	< 5	w
Coolant Flow Rate:	14	L/min
Auxiliary Flow Rate:	0.8	L/min
Nebulizer Flow Rate:	0.4	L/min

Nebulizer Pressure:	34 psi
Sample Delivery Rate:	1.0 mL/min
Viewing Height:	15 mm
Slit Width:	25 µm
Slit Height:	8 mm
Camera Temperature:	-40 °C
Mask:	mem24

2) Peak Definition File:

(Peak Number 1 of 2)

Element and Line:	Ca1
Wavelength:	317.933
Expected Peak Pixel:	331
Range Left:	10
Range Right:	10
Actual Peak Pixel:	330
Background Correction:	ON
Type:	TWO SIDE
Rel Strt Lo:	-20
Range:	8
Rel Strt Hi:	12
Range:	8
Peak:	AREA
Type:	FIXED
Rel Low:	5
Rel High:	5

(Peak Number 2 of 2)

Element and Line:	Mg3
Wavelength:	279.553
Expected Peak Pixel:	29
Range Left:	10
Range Right:	10
Actual Peak Pixel:	27
Background Correction:	ON
Type:	TWO SIDE
Rel Strt Lo:	-20
Range:	8
Rel Strt Hi:	12
Range:	8
Peak:	AREA
Type:	FIXED
Rel Low:	5
Rel High:	5

3) Task Definition File:

Mask:	mem24
ID:	Mg3
Integration Time:	2.000
Mode:	sim
ID:	Ca1
Integration Time:	2.000
Mode:	sim

Calibration Data:

Mg3 Slope:	8215.15
Intercept:	27.05
Curvature:	73.238
log-log slope:	1.00989
Ca1 Slope:	355.59
Intercept:	1.99
Curvature:	1.086
log-log slope:	1.00733
Warmup Period:	30 min
Clearing Time:	50 s
Signal to Noise Ratio Threshold:	500.0
Sample Dilution Factor:	1.0
Calibration Order:	(B,STD,STD,STD,STD,STD)
Analysis Order:	(B,STD,35S)
Sample Integration time:	2.000 s
Number of Repetitions:	5
Elements:	Ca, Mg
High STD Integration time:	2.000 s
Number of Repetitions:	5
Mg (High Standard):	3.889 ppm
Ca (High Standard):	12.83 ppm
Low STD Integration Time:	2.000 s
Number of Repetitions:	5
Mg (Low Standard):	0.389 ppm
Ca (Low Standard):	1.283 ppm

The concentrations of sodium and potassium were determined on a Varian SpectrAA-10 atomic absorption spectrophotometer under the following conditions.

Instrument Mode:	Flame Emission
Calibration Mode:	Concentration
Measurement Mode:	Integration
Sample Introduction:	Manual
Delay Time:	0
Measurement Time:	3 s
Replicates:	5
Flame:	Air-Acetylene
Air Flow Rate:	3 L/min
Acetylene Flow Rate:	1.5 L/min
Photomultiplier Voltage:	about 400 V
Na Wavelength:	589.0 nm
Slit Width:	0.5 nm
K Wavelength:	766.5 nm
Slit Width:	1.0 nm

3.3.1 Calibration Standards for Determination of Calcium and Magnesium by ICP

Five standards were prepared in 25-mL volumetric flasks with the following concentrations:

Flask:	1	2	3	4	5
Ca (ppm):	1.28	3.85	6.41	9.62	12.83
Mg (ppm):	0.389	0.778	1.56	3.11	3.89

Using the instrumental conditions stated above for the Leco ICP, sodium and potassium at concentrations of less than 10 ppm do not interfere with the determination of calcium and magnesium, and linear calibration plots are obtained over the concentration range used.

3.3.2 Calibration Standards for the Determination of Sodium and Potassium by Flame Atomic Emission

Five standards were prepared in 25-mL flasks, with the following concentrations:

Flask:	1	2	3	4	5
Na (ppm):	0.92	1.84	3.68	7.36	9.20
K (ppm):	12.51	9.38	6.26	3.13	1.56

Each of these standards as well as all samples were made 0.04 M in $LiNO_3$ to serve as ion suppressor. With the instrument burner head in the normal position, calibration lines were observed to curve downward because of self-absorption by atoms in the flame path. This effect could be reduced significantly by turning the burner head 90 degrees to reduce the path length. Linear calibration plots were obtained by this method.

3.4 *Procedure for Loading and Eluting the Resin Column*

The procedure for operation of the resin column, as described in reference [16], consists of five steps: 1. Equilibration with the solution to be analyzed by passage through the column until eluent from the column has the same concentration as the original solution. 2. Passage of a segment of air through the column to displace most of the residual solution. 3. Passage of pure water through the column to wash out the last portions of solution remaining in the column. 4. Elution of the metals sorbed on the column with 2 M HNO_3. 5. A final wash with pure water to remove nitric acid eluent from the column and connecting tubing and to prepare the column for another sample.

For applications of the column described above to solutions containing the normal concentrations of sodium, potassium, calcium and magnesium found in urine samples, preliminary studies showed that 15 minutes is adequate for equilibration and 2 minutes is enough for elution. For the studies in this chapter, 15 to 30 minutes were used as the equilibration time, depending on the degree of resin swelling caused by different compositions of sample solutions. Usually a sample volume of 25 mL was passed through the column, followed by air for about 3 minutes, then a 3 to 5 minute back wash with pure water, a 3 minute elution of the metal ions exchanged in the resin phase with 2 M HNO_3, and a final water wash for 3 minutes. The solutions from the last two steps were collected in a 100-mL volumetric flask. After dilution of the flask contents to volume with pure water the concentrations of calcium and magnesium in the elution flask were determined by ICP-AES. For the determination of sodium and potassium in the elution flask, a 10-mL aliquot of the original 100 mL of solution was transferred to a 25-mL volumetric flask, 1 mL of 1 M $LiNO_3$ added, and the contents diluted to volume with water. This solution was then analyzed for sodium and potassium concentrations by flame AES with the Varian SpectrAA-10 atomic absorption spectrophotometer.

For the determination of total concentrations of sodium, potassium, calcium and magnesium in urine samples, a 100 times dilution was performed before calcium and magnesium were measured by ICP-AES, and a 1000 times dilution was needed for sodium and potassium determination by flame AES with the Varian SpectrAA-10 atomic absorption spectrophotometer. $LiNO_3$ was added to give the same final concentration of lithium ion as for the standards.

3.5 *Theory of the Method*

The column equilibration method is suitable for speciation of polyvalent metal ions in kinetically labile systems. The original method is based on equilibration of a small resin column with a sample solution under trace conditions, that is, where the metal ion under study is sorbed to less than one percent of the total column capacity. We are interested in application of the method under non-trace conditions, where enough of the ion of interest is sorbed on the resin to produce highly non-linear calibration plots.

Consider the equilibration of a strong-acid type cation exchange resin with a solution containing a polyvalent metal ion M^{n+} and an electrolyte such as $NaNO_3$. For this system the equilibrium reaction is (R^- = resin phase exchange sites):

$$M^{n+} + nRNa \rightleftharpoons RnM + nNa^+ \qquad (1)$$

The corresponding thermodynamic equilibrium constant K is:

$$K = \frac{a_{R_nM} \cdot a^n_{Na^+}}{a_{M^{n+}} . a^n_{RNa}} = \frac{[R_nM][Na^+]^n}{[M^{n+}][RNa]^n} \cdot \frac{\gamma_{R_nM} \cdot \gamma^n_{Na^+}}{\gamma_{M^{n+}} \cdot \gamma^n_{RNa}} \qquad (2)$$

If the univalent ion concentration is kept constant (that is, $[Na^+]$ is held constant) and large relative to M^{n+}, so that the fraction of resin in the R_nM form is less than 1% of the resin capacity C (here $C = [RNa] + n[R_nM]$), the activity coefficients and [RNa] in equation (2) become essentially constant, and $[M^{n+}]$ becomes directly proportional to $[R_nM]$. This is defined as trace ion exchange conditions. Trace conditions are typically maintained by providing a high concentration of univalent cations in the solution phase.

Ideally a series of solutions under investigation would contain the same amount of electrolyte meeting trace ion exchange conditions so that a single linear calibration curve would be enough to cover the unknown samples under study. But in many real systems, such as urine samples, the electrolyte concentration will vary from sample to sample and may not be high enough to fit trace conditions. If the concentration of electrolyte in the samples does satisfy trace conditions but varies from sample to sample, then a set of calibration curves are needed, with each curve being constructed at a specified level of electrolyte concentration. This is inconvenient in practice.

If electrolyte concentrations in the solutions under study are not high enough to fit trace conditions, more electrolyte could be added to bring them to a level where one calibration curve could be used. But here two disadvantageous things happen. One is inconvenience, in that samples cannot be run through the column until the electrolyte concentration is measured and adjusted. This delay may cause problems in clinical studies, for example, because components of urine samples often precipitate out on standing for more than 2 hours or so. The second disadvantage is that the original equilibrium might be disturbed by the addition of electrolyte simply because the ionic strength is shifted. In order to obtain accurate free metal ion concentrations in the original system, changes to the original sample should be avoided as much as possible. This means that it would be much more useful if the ion-exchange equilibration method could be applied under non-trace conditions.

A previous study [16] described an approach for the determination of free calcium and magnesium in urine samples under non-trace conditions. The method used was to prepare calibration curves using differing amounts of calcium and magnesium while holding the amounts of sodium and potassium constant at levels corresponding to those present in the NIST standard reference material SRM 2670, Freeze Dried Urine. After measuring the amounts of calcium and magnesium on the resin, [R_2Ca] and [R_2Mg], by atomic absorption, an empirical expression was obtained by fitting the following equations by multiple regression:

$$[R_2Ca]^2 = a[Ca] + b[Ca][Mg] \tag{3}$$

$$[R_2Mg]^2 = p[Ca] + q[Mg] + r[Ca]^2 + s[Mg]^2 + t[Ca][Mg] \tag{4}$$

In these equations the coefficients a, b, p, q, r, s and t must be determined for each of the free metal ion concentration terms.

This method is applicable to samples like the NIST reference material SRM 2670, where sodium and potassium levels are known in advance, but cannot be considered a candidate for the routine determination of free calcium and magnesium owing to the necessity for close matching of samples and standards in terms of sodium and potassium concentrations. Since potassium and sodium levels in urine vary from sample to sample and are not known in advance, preparation of standards prior to the analysis of an unknown urine is not possible. Also, urine samples cannot be stored for more than about 2 hours prior to analysis owing to decomposition and precipitation of components, making the determination of free calcium and free magnesium based on standards prepared after obtaining the sample unsatisfactory for routine work. Therefore a general method is needed which can measure free metal ion concentrations under non-trace conditions and which does not require the user to match standards and samples in terms of sodium and potassium concentrations.

From equation (2) we know that the thermodynamic column equilibrium constant consists of a concentration term and an activity coefficient term. The value should be a constant at constant temperature. In this equation $[M^{n+}]$ is the concentration of free metal ion we are seeking, $[Na^+]$ is the free sodium concentration (which under many conditions is close to the total sodium concentration in solution), and $[R_nM]$ and [RNa] are the concentrations of metal ion of interest and sodium ion sorbed in the resin phase. (The latter two concentrations can be determined using ICP-AES or flame AES.) The terms $\gamma_{M^{n+}}$ and γ_{Na^+} are the activity coefficients of the metal ion and sodium ion, respectively, in the solution phase; their values are determined by the ionic strength of the solution.

A major difficulty here is the determination of γ_{R_nM} and γ_{RNa}, the activity coefficients of the metal ion of interest and sodium ion in the resin phase. This difficulty holds true for all theoretical treatments of resin-solution equilibria, and no practical method is available for the measurement of these activity coefficients [133]. Theoretical estimates may be in error as much as 200% or more, which is unsuitable to routine use.

Since the major cations in urine are Na^+, K^+, Ca^{2+} and Mg^{2+}, a series of synthetic samples containing the nitrate salts of these four ions was employed in the initial development of the method described here. In these initial solutions no complexing ligands were added, so the free metal ion concentrations were the same as the total metal ion concentrations.

The resin used was a strong acidic cation exchanger, Amberlyst 15, previously converted to the hydrogen form. When a synthetic sample is passed through the column, all four ions will exchange with the hydrogen ions in the resin phase. Since the pH of the synthetic samples was around 7 and the resin is a strongly acidic type, after equilibrium the major components on the resin phase are expected to be R_2Ca, R_2Mg, RNa and RK, and the total capacity of the resin is equal to

$$\text{Total Capacity} = [RNa] + [RK] + 2[R_2Ca] + 2[R_2Mg] \tag{5}$$

Here four equilibria exist simultaneously:

$$Ca^{2+} + 2RNa \rightleftharpoons R_2Ca + 2Na^+ \tag{6}$$

$$Mg^{2+} + 2RNa \rightleftharpoons R_2Mg + 2Na^+ \tag{7}$$

$$Ca^{2+} + 2RK \rightleftharpoons R_2Ca + 2K^+ \tag{8}$$

$$Mg^{2+} + 2RK \rightleftharpoons R_2Mg + 2K^+ \tag{9}$$

with four corresponding thermodynamic equilibrium constants:

$$K_{Ca/Na} = \frac{[R_2Ca][Na^+]^2}{[Ca^{2+}][RNa]^2} \cdot \frac{\gamma_{R_2Ca} \cdot \gamma^2_{Na^+}}{\gamma_{Ca^{2+}} \cdot \gamma^2_{RNa}} \tag{10}$$

$$K_{Mg/Na} = \frac{[R_2Mg][Na^+]^2}{[Mg^{2+}][RNa]^2} \cdot \frac{\gamma_{R_2Mg} \cdot \gamma^2_{Na^+}}{\gamma_{Mg^{2+}} \cdot \gamma^2_{RNa}} \tag{11}$$

$$K_{Ca/K} = \frac{[R_2Ca][K^+]^2}{[Ca^{2+}][RK]^2} \cdot \frac{\gamma_{R_2Ca} \cdot \gamma^2_{K^+}}{\gamma_{Ca^{2+}} \cdot \gamma^2_{RK}} \tag{12}$$

$$K_{Mg/K} = \frac{[R_2Mg][K^+]^2}{[Mg^{2+}][RK]^2} \cdot \frac{\gamma_{R_2Mg} \cdot \gamma^2_{K^+}}{\gamma_{Mg^{2+}} \cdot \gamma^2_{RK}} \tag{13}$$

Although these constants should not vary at constant temperature, the activity coefficients in the resin phase are not easy to determine. After some preliminary experiments and calculations, it was found possible to show that the ratio of the activity coefficients in the resin phase could be related to [RNa], [RK], [R_2Ca], and [R_2Mg], the concentrations of metal ion species present in the resin phase. This is reasonable since the composition in the resin phase is the major factor determining the resin phase properties, including activity coefficients in that phase. It now became possible to define a mixed constant K^{mix} for each pair of ions in the system that includes concentration terms and activity coefficients in the solution phase:

$$K^{mix}_{Ca/Na} = \frac{[R_2Ca][Na^+]^2}{[Ca^{2+}][RNa]^2} \cdot \frac{\gamma^2_{Na^+}}{\gamma_{Ca^{2+}}} = K_{Ca/Na} \cdot \frac{\gamma^2_{RNa}}{\gamma_{R_2Ca}} \qquad (14)$$

$$K^{mix}_{Mg/Na} = \frac{[R_2Mg][Na^+]^2}{[Mg^{2+}][RNa]^2} \cdot \frac{\gamma^2_{Na^+}}{\gamma_{Mg^{2+}}} = K_{Mg/Na} \cdot \frac{\gamma^2_{RNa}}{\gamma_{R_2Mg}} \qquad (15)$$

$$K^{mix}_{Ca/K} = \frac{[R_2Ca][K^+]^2}{[Ca^{2+}][RK]^2} \cdot \frac{\gamma^2_{K^+}}{\gamma_{Ca^{2+}}} = K_{Ca/K} \cdot \frac{\gamma^2_{RK}}{\gamma_{R_2Ca}} \qquad (16)$$

$$K^{mix}_{Mg/K} = \frac{[R_2Mg][K^+]^2}{[Mg^{2+}][RK]^2} \cdot \frac{\gamma^2_{K^+}}{\gamma_{Mg^{2+}}} = K_{Mg/K} \cdot \frac{\gamma^2_{RK}}{\gamma_{R_2Mg}} \qquad (17)$$

As mentioned earlier, it is difficult to determine the activity coefficients in the resin phase, but the ratio of them can be estimated. For Amberlyst 15 resin studied here, the mixed constants in equations (14) to (17) can be related to the composition of the resin phase. Through trial and error, it was found that the best predictions of new mixed constants could be obtained by a linear relation between the log values of the mixed constants and the ion composition in the resin phase. The equations corresponding to these relations are:

$$\mathrm{Log}(K^{mix}{}_{Ca/Na}) = a1[RK] + b1[R_2Ca] + c1[R_2Mg] + d1 \qquad (18)$$

$$\mathrm{Log}(K^{mix}{}_{Mg/Na}) = a2[RK] + b2[R_2Ca] + c2[R_2Mg] + d2 \qquad (19)$$

$$\mathrm{Log}(K^{mix}{}_{Ca/K}) = a3[RK] + b3[R_2Ca] + c3[R_2Mg] + d3 \qquad (20)$$

$$\mathrm{Log}(K^{mix}_{Mg/K}) = a4[RK] + b4[R_2Ca] + c4[R_2Mg] + d4 \qquad (21)$$

where a1, a2, a3, a4, b1, b2, b3, b4, c1, c2, c3, c4, d1, d2, d3, and d4 are the coefficients obtained by fitting the above equations using the method described in the next section. Although there are four ionic components in the resin phase, only three of them were used in the above equations because the total capacity of the column is fixed, so that the fourth variable is not independent. Once these coefficients have been obtained for a particular column, they can be used to predict new mixed constants for unknown solutions passed through that column.

In the special situation where the concentrations of Na^+ and K^+ are much higher than those of Ca^{2+} and Mg^{2+}, the ionic strength of the solution can be estimated by assuming that the Na^+ and K^+ concentrations control the ionic strength, that is,

$$\mu = [Na^+] + [K^+] \qquad (22)$$

and the activity coefficients in solution which appear in equations (14) to (17) could be calculated using the Davies Equation. If the concentrations of ligands capable of forming complexes with Na^+ and K^+ are not high, then another approximation can be made, that is, the free Na^+ and K^+ concentrations are nearly equal to the total Na^+ and K^+ concentrations in solution. Also, values of [RNa], [RK], $[R_2Ca]$ and $[R_2Mg]$ can be determined by chemical analysis using methods such as ICP-AES or flame-AES. With this information, we see upon examination of equations (14) to (17) that the free calcium concentration can be determined using equations (14) and (16), and the free magnesium concentration using equations (15) and (17). This allows two values to be calculated for calcium and two for magnesium, one pair through the mixed constant related to sodium, and another pair through the constant related to potassium. By taking the average of the two results for each ion the final value obtained will have higher precision than the use of only one result.

3.6 *Calculation of Coefficients Relating Log Values of Mixed Constants to the Composition of the Resin Phase*

As mentioned in the previous section, to predict new mixed constants for unknown samples the coefficients which relate log values of mixed constants to the composition of the resin phase must be known for a particular column. This can be achieved by calibrating the column with a group of standard solutions which contain cation species at levels similar to those of the samples under investigation. The standards were prepared with a non-complexing anion so that the free cation concentrations could be considered to be the same as the total cation concentrations.

For each standard solution, four mixed constants ($K^{mix}{}_{Ca/Na}$, $K^{mix}{}_{Mg/Na}$, $K^{mix}{}_{Ca/K}$, and $K^{mix}{}_{Mg/K}$) can be calculated as defined in equations (14) to (17). Also, $[R_nM]$ values are available from ICP-AES or flame-AES analyses. Expressions of the form shown in equations (18) to (21) can then be written with the coefficients a, b, c, and d as the unknowns. In theory, four standard solutions are enough to determine the four coefficients for each of the mixed constants, but in practice the preparation of more standard solutions is recommended to provide a good representation of the different combinations of cation concentrations that may occur, and to reduce experimental errors in the values calculated for the coefficients.

When the number of equations (here equal to the number of standard solutions) is larger than the number of unknowns (here, a, b, c, and d), a matrix method is useful. To apply such a method we define the log value of the mixed constant matrix as K. Then K is equal to

$$\begin{bmatrix} \log(K^{mix}_{Ca/Na})1 & \log(K^{mix}_{Mg/Na})1 & \log(K^{mix}_{Ca/K})1 & \log(K^{mix}_{Mg/K})1 \\ \log(K^{mix}_{Ca/Na})2 & \log(K^{mix}_{Mg/Na})2 & \log(K^{mix}_{Ca/K})2 & \log(K^{mix}_{Mg/K})2 \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & \cdots\cdot \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & \cdots\cdot \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & \cdots\cdot \\ \log(K^{mix}_{Ca/Na})n & \log(K^{mix}_{Mg/Na})n & \log(K^{mix}_{Ca/K})n & \log(K^{mix}_{Mg/K})n \end{bmatrix} \tag{23}$$

where the first-row components are the log values of mixed constants obtained from standard 1, the second-row values from standard 2, and so on to the nth row from standard n. Similarly, the matrix for the concentration in the resin phase can be defined as R, where R is equal to

$$\begin{bmatrix} [RK]1 & [R_2Ca]1 & [R_2Mg]1 & 1 \\ [RK]2 & [R_2Ca]2 & [R_2Mg]2 & 1 \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & 1 \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & 1 \\ \cdots\cdot & \cdots\cdot & \cdots\cdot & 1 \\ [RK]n & [R_2Ca]n & [R_2Mg]n & 1 \end{bmatrix} \tag{24}$$

Here the first-row components are the concentrations of cations absorbed on the resin phase from standard 1, the second row the concentrations from standard 2, and so on to the nth row from standard n. The last component of each row is 1, which when multiplied by d equals d. Here the concentration unit can be chosen arbitrarily so long as the units for the calibration standards and samples are the same. In this study, micromoles of each cation in the column were used as the concentration units.

Also, a coefficients matrix, C, can be defined as

$$\begin{bmatrix} a1 & a2 & a3 & a4 \\ b1 & b2 & b3 & b4 \\ c1 & c2 & c3 & c4 \\ d1 & d2 & d3 & d4 \end{bmatrix} \quad (25)$$

where the first column components are coefficients related to the Ca/Na mixed constant, the second column to the Mg/Na mixed constant, the third to the Ca/K mixed constant and the last to the Mg/K mixed constant, as defined in equations (18) to (21).

Using the above definition, equivalent expressions of equations (18) to (21) for a set of n standards can be written as

$$K = R\,C \quad (26)$$

Here C is the coefficient matrix we are seeking, and K and R are the mixed constant and resin phase concentration matrices obtained from the calibration standards. To obtain C, the relation

$$R^T K = R^T R\,C \quad (27)$$

was used. Here R^T is the transposed matrix of R,

$$(R^T R)^{-1} R^T K = (R^T R)^{-1} (R^T R)\,C \quad (28)$$

That is, the coefficient matrix C is

$$C = (R^T R)^{-1} R^T K \quad (29)$$

A program was written in BASIC language to perform this matrix calculation. A listing of the program is provided in Appendix IV.

3.7 *Calibration of the Column -- Determination of the Coefficient Matrix*

For the purpose of calibration of the column constructed in this study, 36 standard solutions were prepared. This number was selected to provide a representation of the concentration range expected for each cation in urine samples, and to give a variety of combinations of concentrations. The nitrate salts of sodium, potassium, calcium and magnesium were used to prepare the standard solutions. As mentioned earlier, the free metal ion concentrations in these solutions were assumed to be the same as the total ion concentrations. The compositions of the 36 standards are shown in Table 3.1.

After pumping each sample solution through the ion exchange column until equilibrium was reached, the cations present on the resin were eluted from the column with 2 M HNO_3 and collected in 100-mL volumetric flasks. The concentrations of each cation were then determined using ICP-AES for calcium and magnesium, and flame-AES for sodium and potassium. So long as the same column is used, the values of $[R_nM]$ may be conveniently defined as μmoles of M present in the column. These values, together with the known free metal ion concentrations in the solution phase, were used to calculate mixed constants for each of the standards. The ionic strengths of the original solutions were calculated as the sum of the sodium and potassium concentrations since the calcium and magnesium concentrations were small enough to be neglected. Activity coefficients in solution were calculated using the Davies equation in the form

$$\log(\gamma_i) = -AZ_i^2\left\{\frac{\sqrt{\mu}}{1-\sqrt{\mu}} - 0.3\mu\right\} \qquad (30)$$

where γ_i is the activity coefficient of ionic species i, A is a constant equal to 0.51 for aqueous solutions at 25°C, Z_i is the charge on species i, and μ is the ionic strength of the solution.

Table 3.1 Concentrations of calibration standard solutions

No.	Na (mM)	K (mM)	Ca (mM)	Mg (mM)
1	50	100	1.6	3.2
2	50	150	4.0	6.4
3	100	50	1.6	6.4
4	100	100	4.0	0.8
5	100	150	0.4	3.2
6	150	100	0.4	6.4
7	150	150	1.6	0.8
8	100	50	1.6	3.2
9	50	100	1.6	0.8
10	50	50	4.0	0.8
11	150	50	1.6	0.8
12	50	50	1.6	6.4
13	150	50	0.4	6.4
14	50	100	0.4	6.4
15	150	150	4.0	3.2
16	150	50	0.4	0.8
17	50	50	0.4	6.4
18	50	50	0.8	1.6
19	100	50	2.0	4.0
20	150	50	4.0	6.4
21	50	100	4.0	4.0
22	100	100	0.8	6.4
23	150	100	2.0	1.6
24	50	150	2.0	6.4
25	100	150	4.0	1.6
26	150	150	0.8	4.0
27	100	20	1.6	1.6
28	200	20	3.2	4.0
29	300	20	4.8	6.4
30	100	60	3.2	6.4
31	200	60	4.8	1.6
32	300	60	1.6	4.0
33	100	100	4.8	4.0
34	200	100	1.6	6.4
35	300	100	3.2	1.6
36	300	100	1.6	1.6

The mixed constants defined in equations (14) to (17) were then calculated for each standard. For these thirty six standards the values for the mixed constants varied from 172 to 336 for Ca/Na, 26 to 98 for Ca/K, 39 to 64 for Mg/Na, and 5.6 to 18 for Mg/K. Clearly the error would be large if one used average values. This dramatic variation is caused, as discussed previously, by differing metal ion concentrations in the resin phase. For example, for this set of standards, sodium occupied 8 to 56% of the total resin capacity, potassium 9 to 60%, calcium 6 to 56%, and magnesium 2 to 33%. The ion exchange equilibrium is clearly functioning here under non-trace conditions. The log values of the mixed constants can be related to the composition of metal ions in the resin phase, as shown in equations (18) to (21). The coefficients were then calculated using equation (29). These coefficients, once obtained by calibration with a set of standards, can be used to predict mixed constants for unknown samples so long as the same column is used. The validity of the method was assessed by comparing recalculated mixed constants for these standards with experimental values using the metal ion concentration in the resin phase and the coefficients. The results are shown in Tables 3.2a, b, c, and d. It can be seen from the data that the predicted values for the mixed constants are reasonably close to the experimental values considering the simple method used, and that the agreement is acceptable for most of the measurements.

As stated above, the coefficients obtained from calibration with a single set of standards were used to calculate a series of mixed constants for unknown samples. From equations (14) to (17), the free [Ca^{2+}] or [Mg^{2+}] can be calculated if values for the mixed constants are available because all terms but free [Ca^{2+}] or [Mg^{2+}] are measurable. A BASIC program was written for this purpose and is provided in Appendix III.

Table 3.2a. Comparison of measured and predicted mixed constant values for the calcium/sodium equilibrium on the resin column used in this work.

No.	Measured	Predicted	No.	Measured	Predicted
1	278	280	2	270	278
3	236	246	4	233	242
5	327	324	6	303	291
7	278	280	8	246	235
9	281	269	10	226	209
11	240	221	12	260	249
13	277	256	13	336	315
15	280	259	16	262	240
17	276	289	18	229	261
19	219	236	20	220	219
21	252	254	22	300	293
23	260	254	24	297	303
25	257	266	26	282	300
27	198	200	28	210	192
29	172	189	30	221	234
31	205	209	32	220	223
33	255	240	34	257	257
35	211	231	36	223	241

Table 3.2b. Comparison of measured and predicted mixed constant values for the calcium/potassium equilibrium on the resin column used in this work.

No.	Measured	Predicted	No.	Measured	Predicted
1	58	60	2	58	59
3	36	40	4	45	50
5	98	104	6	70	73
7	80	83	8	41	41
9	64	63	10	31	30
11	46	45	12	38	36
13	54	50	13	79	71
15	67	60	16	62	61
17	45	48	18	44	48
19	35	39	20	33	33
21	45	45	22	68	65
23	62	59	24	73	69
25	62	63	26	82	89
27	26	28	28	28	29
29	27	29	30	36	36
31	38	37	32	52	48
33	49	42	34	64	57
35	55	55	36	61	64

Table 3.2c Comparison of measured and predicted mixed constant values for the magnesium/sodium equilibrium on the resin column used in this work.

No.	Measured	Predicted	No.	Measured	Predicted
1	59	58	2	61	60
3	46	47	4	59	58
5	58	63	6	51	52
7	63	62	8	51	49
9	62	62	10	53	54
11	51	51	12	49	47
13	46	44	13	53	54
15	64	58	16	51	50
17	45	45	18	50	54
19	46	49	20	48	47
21	57	57	22	54	53
23	56	57	24	62	60
25	63	62	26	56	59
27	42	45	28	44	42
29	39	41	30	48	49
31	47	50	32	47	45
33	59	55	34	53	50
35	53	51	36	50	52

Table 3.2d Comparison of measured and predicted mixed constant values for the magnesium/potassium equilibrium on the resin column used in this work.

No.	Measured	Predicted	No.	Measured	Predicted
1	12.2	12.5	2	13.1	12.6
3	7.0	7.7	4	11.5	12.1
5	17.3	20.1	6	11.8	13.2
7	18.0	18.5	8	8.6	8.4
9	14.1	14.6	10	7.3	7.8
11	9.7	10.3	12	7.2	6.9
13	8.9	8.6	13	12.4	12.1
15	15.5	13.4	16	12.2	12.7
17	7.4	7.6	18	9.6	10.0
19	7.4	8.1	20	7.3	7.0
21	10.2	10.1	22	12.4	11.8
23	13.2	13.3	24	15.1	13.8
25	15.1	14.7	26	16.3	17.5
27	5.6	6.4	28	5.9	6.2
29	6.2	6.2	30	7.8	7.6
31	8.7	8.8	32	11.1	9.9
33	11.4	9.6	34	13.1	11.3
35	13.6	12.2	36	13.5	13.7

3.8 *Application of Method to the Determination of Free Calcium and Magnesium in a Test Solution containing Citrate as Complexing Ligand*

Citrate is one of the important ligand species found in urine. In order to test the method developed in this study, a system containing citrate, calcium and magnesium was investigated to determine the levels of precision and accuracy that could be achieved.

3.8.1 Chemicals and Solutions

Citrate: 0.125 M. Weigh 4.0552 g $K_3C_6H_5O_7 \cdot H_2O$ (F.W. 324.41 BDH Chemicals, Lot No. 82554-1632, Analytical Reagent), dissolve in about 60 mL of water, and adjust the pH from its initial value (9 in this work) to 6.5 with dilute HNO_3 (0.1 M), using a pH meter as a monitor. Dilute to 100 mL in a volumetric flask.

Biobuffer: Bis-tris, 0.095 M, pH 6.5. Weigh 5.23 g Bis-tris (F.W. 209.2, Sigma, Lot No. B-9754) and dissolve in 250 mL water. Using 2 M HNO_3, adjust the pH from 9.4 to 6.5 with a pH meter. About 15 mL of acid is required.

3.8.2 Experimental Procedure

Sample solutions containing different amounts of sodium, potassium, calcium and magnesium nitrate were prepared, and varying amounts of citrate solution added to each, along with 1 mL of biobuffer. Each solution was brought to a volume of 25 mL, the pH recorded, and the solution pumped through the Amberlyst resin column until equilibrium with the resin phase had been attained, about 20 minutes. The resin was then washed as described previously, the sorbed metals eluted from the column with 2 M HNO_3, and the eluate analyzed for calcium, magnesium, sodium, and magnesium by ICP-AES or flame-AES.

3.8.3 Calculation of Free Metal Ion Concentrations in the Test System from Total Metal and Ligand Concentrations plus the Metal-Ligand Stability Constants

A BASIC program was written to calculate the free metal ion concentrations for a system where the total metal ion and ligand concentrations are known. The program could be used for systems containing any number or kind of metal ions and ligands as long as no precipitates or gases formed and the necessary stability constants were available. The stability constants used were taken from the literature[16, 138, 145, 146], and are listed in Table 3.3.

In the program, the conditional stability constants corresponding to the ionic strength of the solution are first calculated so that it is not necessary to input new conditional stability constants for each new solution. This is especially convenient for the system being studied here since the ionic strength varies from one solution to another. The pH of the solution is then entered for each solution to allow calculation of the fraction of each ligand present in the various protonated forms (L, HL, H_2L...). An iteration method is next used to find the equilibrium concentrations of the species by first assuming all the ligands are free (that is, have not complexed with any metal ions), then calculating in sequence the concentration of each form of ligand, the fraction of each metal ion present in the free form, and lastly the amount of uncomplexed ligand present. The latter value is compared with the value found in the previous cycle to determine the adjustment factor, that is, the ratio of uncomplexed to total ligand. A flow chart of the program is shown in Figure 3.2, and the BASIC program used for the calculations is listed in Appendix II.

3.8.4 Results and Discussion

Samples containing different amounts of the four metal ions under study (Na^+, K^+, Ca^{2+}, Mg^{2+}), along with citrate as ligand, were prepared, and the free metal ion concentrations determined by the procedure described above. The composition and pH of each of the test samples are given in Table 3.4.

Because potassium citrate was used as the source of the citrate ligand, the total potassium concentrations used in the calculations were the sum of potassium nitrate and potassium citrate present in each solution. The free sodium and potassium concentrations in solution were considered to be equal to the total sodium and total potassium concentrations. This was confirmed through calculations using literature values for association constants of these ions with nitrate and citrate, which showed that over 99.7% of each was in the free form in the solutions used in this work. Using these free sodium and potassium concentrations, along with the results obtained from analyses of the column eluent ($[R_nM]$), mixture constants could be predicted and the free Ca^{2+} and Mg^{2+} concentrations in solution calculated. The results of these calculations are shown in Table 3.5.

From these results we can see that the experimentally determined values are quite consistent, with averages somewhat higher than values calculated from the initial concentrations. However, the agreement is in general within 10%, which is quite competitive with other methods for free metal ion measurements.

Table 3.3 List of stability constants used in the calculation of free Ca^{2+} and free Mg^{2+} concentrations in presence of citrate. Charges on species omitted for convenience; the triply charged citrate ion is denoted by L. (Values from Stability Constants, Martell, A.E. and Smith, R.M., Vol. 3, Plenum Press, New York, 1977, and Stability Constants of Metal-Ion Complexes, Part B, Organic Ligands, IUPAC, Pergamon Press, Oxford, 1982.)

EQUILIBRIUM	LOG K (μ, temp in °C)
CaL/Ca·L	4.68 (0, 25)
CaHL/Ca·HL	3.09 (0, 25)
$CaH_2L/Ca{\cdot}H_2L$	1.10 (0, 25)
MgL/Mg·L	3.37 (0.1, 25)
MgHL/Mg·HL	1.92 (0.1, 25)
$MgH_2L/Mg{\cdot}H_2L$	0.84 (0.1, 25)
KL/K·L	0.56 (0.15, 37)
KHL/K·HL	-0.30 (0.15, 37)
NaL/Na·L	0.70 (0.1, 25)
NaHL/Na·HL	0.10 (0.15, 37)
HL/H·L	6.396 (0, 25)
$H_2L/H{\cdot}HL$	4.761 (0, 25)
$H_3L/H{\cdot}H_2L$	3.128 (0, 25)

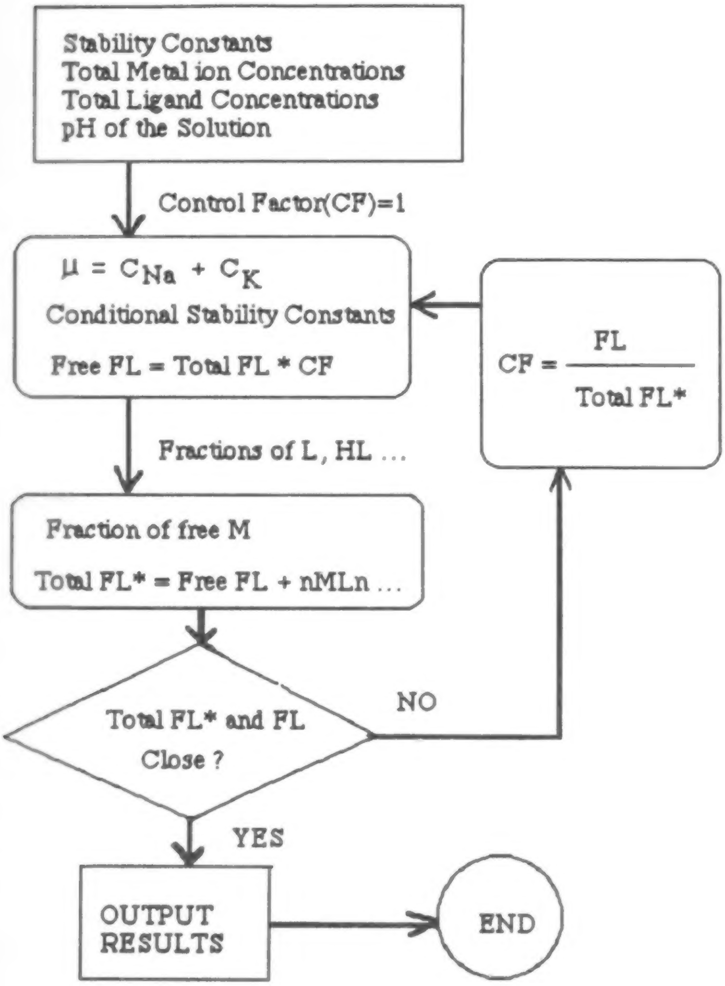

Stability Constants
Total Metal ion Concentrations
Total Ligand Concentrations
pH of the Solution
Control Factor(CF)=1
μ = C_Na + C_K
Conditional Stability Constants
Free FL = Total FL * CF
CF = FL / Total FL*
Fractions of L, HL ...
Fraction of free M
Total FL* = Free FL + nMLn ...
Total FL* and FL Close ?
NO
YES
OUTPUT RESULTS
END

Figure 3.2 Flow chart for calculation of equilibrium species concentrations in a solution where pH, total metal ion concentrations, and total ligand concentrations are known. The BASIC program used to perform these calculations is listed in Appendix II.

Table 3.4 . Composition (total concentration) and pH of synthesized samples used to test the proposed method for determination of free metal ion concentrations.(all concentration terms are in mM). L denotes the citrate anion.

No.	$NaNO_3$	KNO_3	$Ca(NO_3)_2$	$Mg(NO_3)_2$	K_3L	pH
1	100	40	3.2	4.0	1.0	6.34
2	100	40	3.2	6.0	5.0	6.25
3	200	40	3.2	4.0	3.0	6.30
4	200	40	4.8	4.0	5.0	6.20
5	100	100	4.8	6.0	3.0	6.21
6	100	100	3.2	4.0	5.0	6.26
7	200	100	3.2	6.0	3.0	6.26
8	200	100	4.8	6.0	5.0	6.16

Table 3.5 Comparison of calculated and experimental results for the determination of free calcium and magnesium in solutions containing citrate. All concentrations are in units of millimoles per liter.

Ca^{2+} found (calc'd by Na)	Ca^{2+} found (calc'd by K)	Mean Ca^{2+} found, expt'l	Calculated Ca^{2+}	Difference, in %
3.0	3.0	3.0	2.8	7
2.0	2.1	2.0	1.8	10
2.3	2.5	2.4	2.2	8
3.0	3.1	3.1	2.8	10
4.0	4.1	4.0	3.6	10
1.7	1.8	1.7	1.6	6
2.5	2.6	2.6	2.4	8
3.3	3.4	3.4	3.1	9

Mg^{2+} found (calc'd by Na)	Mg^{2+} found (calc'd by K)	Mean Mg^{2+} found, expt'l	Calculated Mg^{2+}	Difference, in %
3.6	3.6	3.6	3.5	3
3.5	3.8	3.7	3.4	8
2.9	3.1	3.0	2.8	7
2.4	2.6	2.5	2.4	4
5.0	5.1	5.0	4.6	8
2.0	2.1	2.1	2.1	0
4.8	4.8	4.8	4.5	6
4.0	4.2	4.1	3.9	5

3.9 *Conclusions*

In this chapter, a calibration procedure for ion exchange equilibration under non-trace conditions has been developed and applied to free calcium and magnesium measurement in the presence of citrate as complexing ligand. The method can accommodate samples and standards of different ionic strengths and pH values within a defined range. Calcium and magnesium concentrations were determined by ICP-AES, while flame AES was used to measure sodium and potassium in the column eluates.

The need to use two different analytical techniques to determine the metals in the column eluate is somewhat slow and inconvenient. It would be much more efficient to be able to determine all of the metals by a single method. The next chapter describes the use of an ARL 34000 ICP-AES instrument to measure all four of the metals - sodium, potassium, calcium and magnesium - simultaneously on a single column eluate.

Chapter 4 Determination of Free Calcium and Magnesium Using Amberlyst 15 Resin and ICP-AES Direct Reader Detection

4.1 *Introduction*

In chapter 3 a method for the determination of ionic calcium and magnesium at the millimolar level in solutions such as urine was described which required knowledge of the total calcium, magnesium, sodium and potassium concentrations in the solution. For that study the total calcium and magnesium in solution were measured using a Leco Plasmarray ICP spectrometer. The concentrations of total sodium and potassium were measured using flame atomic emission spectroscopy because the Leco instrument was not sufficiently sensitive to allow the determination of these metals. The method would be much simpler and more useful if all four of the metals Ca, Mg, Na and K could be determined simultaneously. Fortunately an ARL 34000 direct reading multielement ICP spectrometer became available for use at this point in the project. With this instrument 33 elements can be determined simultaneously, including the 4 elements stated above, along with phosphorus and sulfur. Knowledge of the concentration of these latter two elements could be useful for estimating the amount of phosphate and sulphate in urine samples, which in turn could be used to calculate the percentages of free sodium and potassium in the urines.

The ARL 34000 is based upon a 1 meter Rowland circle with a 1080 line/mm holographic grating on a Paschen-Runge mount. The primary slit is movable and provides limited wavelength scanning capabilities. The spectrometer housing is thermostated, and can be evacuated to permit operation in the vacuum ultraviolet. A maximum of 60 channels can be installed either above or below the spectrometer housing behind metal-dielectric-metal bandpass filters. The filters serve as order sorters and help to reduce stray light.

The ICP source is a 2.5 kW RF generator operating at 27 MHz. The torch assembly is enclosed within a Faraday cage to minimize RF leakage into the environment. The gas controls and the impedance matching network are mounted in the Faraday cage and are accessed from the rear of the instrument. The entire system is interlocked to prevent the plasma from operating when there is insufficient argon flow or when the door to the Faraday cage is open.

Data is collected through a charge transfer ADC and processed by a dedicated Digital Equipment Corporation PDP-11 minicomputer. The PC computer runs under an RT-11 compatible operating system and the application software is written in BASIC and assembler languages. The supported application software is highly interactive and prompts the operator for the appropriate response. The computer controls all operations except introduction of samples after the instrument is turned on.

4.2 *Calibration of the ARL 34000 Spectrometer*

The total ion-exchange capacity, as defined by equation (5), of the column described in the previous chapters is about 100 micro equivalents. After each column equilibration with sample, the column eluent was collected in a 25-mL volumetric flask. For normal urine samples each of the four major metal ions (sodium, potassium, calcium, and magnesium) occupied from 1% to 60% of the total capacity of the column. Therefore the range of concentrations of these ions to be expected in the eluent was known, and ICP standards could be prepared to fit within this range. No spectral interferences between these metal ions were found in any of the standards prepared in this work. All standards were prepared in 200-mL volumetric flasks from mixtures of nitrate salt solutions of each ion. The stock solutions were prepared as described in the previous chapter, except that 20 mL of 2 M HNO_3 were added to each as a preservative. All standard solutions were stored in polyethylene bottles. Linear calibration curves were obtained for all four metals over the concentration ranges of interest in this study, as shown in Figures 4.1 to 4.4.

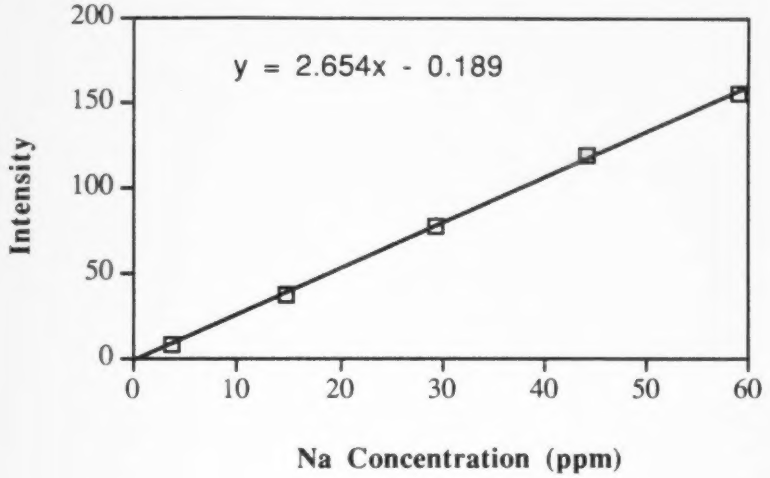


Figure 4.1 Calibration curve for sodium obtained on the ARL 34000 ICP spectrometer.

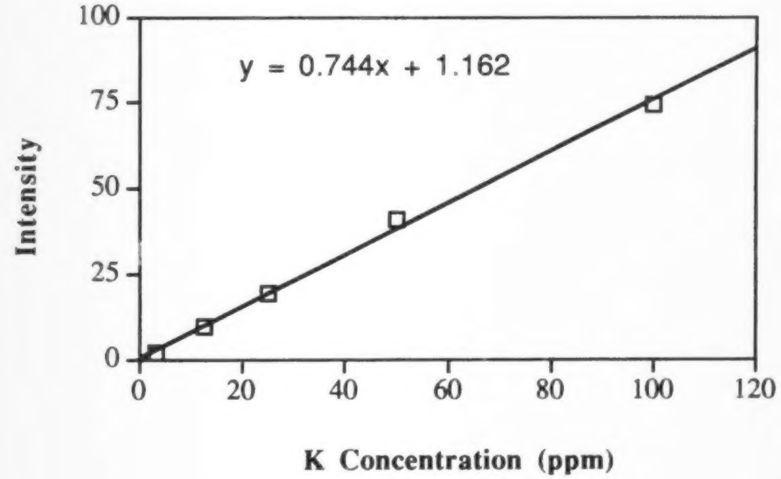


Figure 4.2 Calibration curve for potassium obtained on the ARL 34000 ICP spectrometer.

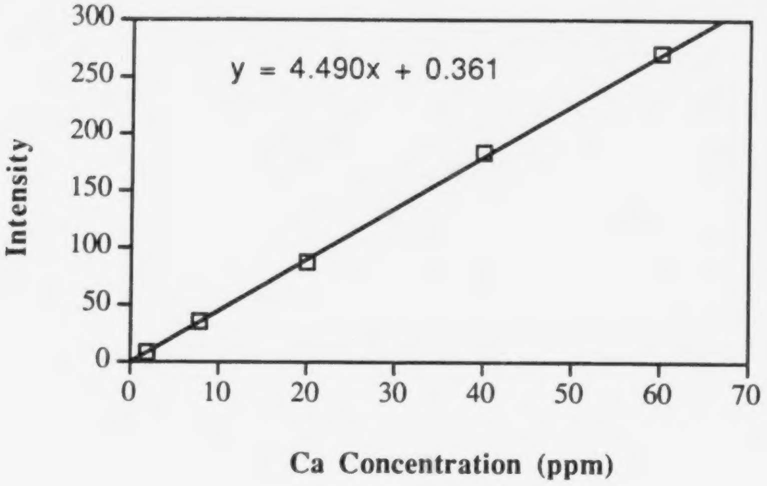


Figure 4.3 Calibration curve for calcium obtained on the ARL 34000 ICP spectrometer.

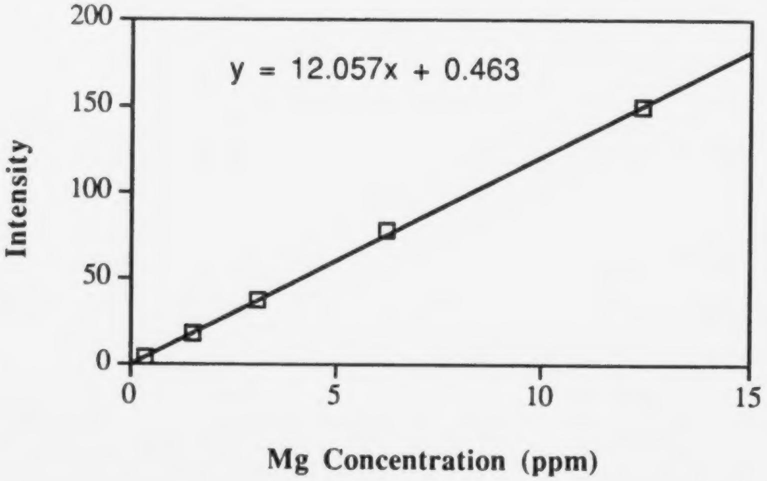


Figure 4.4 Calibration curve for magnesium obtained on the ARL 34000 ICP spectrometer.

4.3 *Calibration of the Column Using ARL 34000 ICP Spectrometer*

Although the same column was used as in the previous chapter, it was decided to recalibrate it because of changes in the ICP detection instrument and in the volume of solution being analyzed. Also, because significant quantities of ammonium ion can be present in urine samples, it was decided to include this ion in some of the standards. Inclusion of ammonium ion in the system has some advantages. One is that potential interference from ammonium ion in the analysis is compensated for by inclusion in the standards. Another is that the precision of the calculations of free calcium and magnesium concentrations is improved because more experimental data can be included in the calculation of the K^{mix} values.

Standards were prepared in 25-mL volumetric flasks from nitrate salts of the four metal ions as in previous work. As mentioned above, ammonium ion was added as ammonium nitrate to some of the standards. The composition of the final set of standards is given in Table 4.1.

After passing each sample through the column until equilibrium was achieved, the cations on the column were eluted with 2 M HNO_3 into a 25-mL volumetric flask. The concentration of each cation was then determined by ICP-atomic emission spectroscopy on the ARL instrument. These concentration values were then used to calculate the mixed constants as described earlier. For calculations of the K^{mix} values the concentration units chosen were micromoles per 25 mL for the ions in the 25-mL elution flask, and millimoles per liter for the metal ions present in the original solution. The ionic strength of the original solution was calculated as the sum of sodium and potassium salt concentrations, assuming them to be present as 1:1 electrolytes. This assumption was considered valid because the concentrations of these two ions were of the order of 20 to 100 times larger than those of calcium and magnesium, the other major cations present in urine. Activity coefficients

Table 4.1 Composition of standard solutions used to calibrate the ion-exchange column. All concentrations are millimoles per liter.

Soln. No.	Na^+	K^+	Ca^{2+}	Mg^{2+}	NH_4^+
1	60	40	1.6	1.6	0
2	60	80	3.2	4.0	0
3	60	120	4.8	6.0	0
4	100	20	1.6	4.0	0
5	100	40	0.8	6.0	0
6	100	80	4.8	0.8	0
7	100	120	3.2	1.6	0
8	200	20	3.2	6.0	0
9	200	40	4.8	4.0	0
10	200	80	0.8	1.6	0
11	200	120	1.6	0.8	0
12	280	20	4.8	1.6	0
13	280	40	3.2	0.8	0
14	280	80	1.6	6.0	0
15	280	120	0.8	4.0	0
16	200	80	3.2	4.0	0
17	100	40	1.6	1.6	20
18	100	40	1.6	1.6	50
19	100	40	1.6	1.6	100

were calculated using the Davies equation .

After the mixed constants K^{mix} for Ca/Na, Ca/K, Mg/Na and Mg/K were calculated for each standard solution, they were related to the composition of the metal ion concentration in the resin phase by means of the following expression:

$$\log(K^{mix}) = a[NaR] + b[KR] + c[CaR_2] + d[MgR_2] + e$$

The coefficient matrix was then calculated by the method described in chapter 3 using all 19 standard solutions. The results are presented in Table 4.2. Using this coefficient matrix and the measured quantities of metal ion eluted from the resin phase, mixed constants for each standard could be calculated. Table 4.3 lists values for these constants, along with the experimental results. It can be seen from Table 4.3 that the predicted K^{mix} values agree well with the experimental values. For the set of 76 values, in only one case does the difference exceed 10%, and in most instances the difference is less than 5%.

4.4 *Application of Method to the Determination of Free Calcium and Magnesium Concentrations in the Presence of Complexing Ligands*

After a column has been calibrated as described in the preceding section, and using the theory developed in the previous chapter, the coefficient matrix can be used to measure free calcium and magnesium concentrations in unknown samples as long as the same column is employed. Systems containing citrate, sulphate, and phosphate, three ligands present in urine, were studied to see whether the free, instead of total, calcium and magnesium concentrations could be measured. The experimentally determined free calcium and magnesium concentrations were then compared with values calculated for these known systems.

Table 4.2 Coefficient matrix for Amberlyst 15 resin column using ARL ICP-AES to determine all four metal ions in the resin phase.

Coeff.	K^{mix}Ca/Na	K^{mix}Ca/K	K^{mix}Mg/Na	K^{mix}Mg/K
a	-0.00118	-0.00676	-0.00222	-0.00780
b	0.00291	0.00150	0.00166	0.000258
c	-0.00489	-0.0257	-0.00333	-0.0241
d	0.000182	-0.0218	-0.00614	-0.0282
e	2.43	2.35	1.86	1.78

Table 4.3a Comparison of predicted and experimental values for $K^{mix}Ca/Na$

Solution. number	$K^{mix}Ca/Na$ predicted	$K^{mix}Ca/Na$ experimental	% difference
1	246	249	- 1.3
2	268	265	1.1
3	283	264	7.4
4	227	229	- 1.1
5	270	273	- 0.9
6	247	266	- 7.3
7	288	292	- 1.1
8	215	218	- 1.2
9	222	227	- 2.2
10	282	292	- 3.4
11	294	292	0.8
12	203	196	3.6
13	223	217	2.7
14	264	271	- 2.7
15	294	290	1.3
16	258	256	0.5
17	243	234	4.2
18	246	238	3.6
19	249	257	- 3.0

Table 4.3b Comparison of predicted and experimental values for $K^{mix}Ca/K$

Solution. number	$K^{mix}Ca/K$ predicted	$K^{mix}Ca/K$ experimental	% difference
1	41.4	40.4	2.5
2	48.5	48.6	-0.1
3	54.7	54.2	0.9
4	31.2	29.9	4.2
5	45.4	45.7	0.6
6	44.8	48.2	-7.1
7	66.7	65.1	2.5
8	31.5	30.8	2.2
9	36.0	36.2	-0.6
10	74.8	72.5	3.1
11	83.2	78.0	6.7
12	32.6	31.4	3.9
13	43.9	44.2	-0.8
14	60.5	67.0	-9.6
15	81.8	81.6	0.2
16	53.1	55.2	-3.8
17	49.6	51.5	-3.8
18	60.8	60.5	0.5
19	75.3	74.4	1.1

Table 4.3c Comparison of predicted and experimental values for $K^{mix}Mg/Na$

Solution. number	$K^{mix}Mg/Na$ predicted	$K^{mix}Mg/Na$ experimental	% difference
1	59.4	57.7	3.0
2	63.0	62.7	0.4
3	65.7	63.9	2.9
4	50.9	50.5	0.7
5	54.0	53.7	0.6
6	62.7	67.2	-6.7
7	68.2	67.6	1.0
8	49.2	49.8	-1.2
9	53.5	55.4	-3.5
10	61.3	60.4	1.5
11	66.1	64.5	2.5
12	49.6	48.5	2.3
13	53.0	50.7	4.5
14	56.3	58.8	-4.2
15	61.5	62.6	-1.7
16	58.7	61.6	-4.7
17	58.3	56.4	3.2
18	59.8	58.2	2.8
19	61.6	63.1	-2.4

Table 4.3d Comparison of predicted and experimental values for $K^{mix}Mg/K$

Solution. number	$K^{mix}Mg/K$ predicted	$K^{mix}Mg/K$ experimental	% difference
1	10.0	9.36	6.8
2	11.4	11.5	-0.9
3	12.7	13.1	-3.4
4	7.00	6.60	6.1
5	9.08	8.99	1.0
6	11.4	12.2	-6.5
7	15.8	15.1	4.7
8	7.20	7.04	2.3
9	8.66	8.83	-2.0
10	16.3	15.0	8.3
11	18.7	17.2	8.5
12	7.98	7.78	2.5
13	10.4	10.3	0.9
14	12.9	14.5	-11
15	17.1	17.6	-2.7
16	12.1	13.2	-8.7
17	11.9	12.5	-4.7
18	14.8	14.8	0
19	18.6	18.3	1.7

4.4.1 Application to a System Containing Citrate as Complexing Ligand

Sample solution were prepared containing different amounts of the nitrate salts of sodium, potassium, calcium, and magnesium, along with potassium citrate as complexing ligand, in 25-mL volumetric flasks. The pH of the samples was measured with a pH meter for use in the calculation of free calcium and magnesium values from stability constants. Each solution was then passed through the calibrated column and eluted with 2 M HNO_3 into a 25-mL flask. The eluent was diluted to 25 mL with water and taken to the ARL 34000 ICP-AES instrument for determination of the amount of each metal ion sorbed on the resin phase. Since the ligand concentrations were small (3 to 6 mM) compared to the sodium and potassium concentrations (40 to 200 mM) in the samples, and also because the stability constants for sodium citrate and potassium citrate are small, the free sodium and potassium concentrations were taken as equal to their total concentrations (calculations showed more than 99.7% of the sodium and potassium to be in the free form). The experimental free calcium and magnesium concentrations were calculated from the coefficient matrix obtained by the calibration of the column, the free sodium and potassium concentrations in each solution (taken as the total concentration for each), and the metal ion concentrations in the resin phase. As a basis for comparison, free calcium and magnesium concentrations were independently calculated from the added total ligand and total metal ion concentrations, the conditional stability constants of all the metal-ligand complexes formed, and the pH of the solutions. The calculated and experimental results are shown in Table 4.4.

From the values in this table we see that the experimental free calcium and magnesium concentrations agree quite well with the calculated values. This confirms that the method can selectively and accurately measure free calcium and magnesium concentrations in the presence of citrate.

4.4.2 Application to a System Containing Sulphate as Complexing Ligand

This experiment was carried out in the same way as for the citrate system except that sulphate replaced citrate as ligand. A 0.5 M Na_2SO_4 solution was prepared by weighing 3.5507 g Na_2SO_4 (F.W. 142.04, Certified ACS, Lot 74203, Fisher Scientific), dissolving the material in H_2O and diluting the solution to 50 mL. A relatively high (0.02 to 0.04 M) concentration of sulphate was selected to bring it close to the range normally found in urine. Because of this high level, complexation of sodium and potassium by the sulphate could not be ignored. Therefore the sulphate concentration in unknown samples was determined by measurement of the sulphur concentration of the sample by ICP-AES and assuming all of the sulphur to be present as sulphate. With knowledge of the sulphate concentration the free sodium and potassium levels were calculated and these values then used for the determination of free calcium and magnesium. The results obtained in this way are shown in Table 4.5.

4.4.3 Application to a System Containing Phosphate as Complexing Ligand

This experiment was carried out in the same way as for sulphate. Here again the phosphate concentrations in urine are relatively high (0.02 to 0.04M) and so complexation with sodium and potassium can not be ignored. Therefore a 0.5 M K_2HPO_4 solution was prepared by dissolving 8.7103g K_2HPO_4 (F.W. 174.18, Lot 880597, Fisher Scientific) in 60 mL H_2O, adjusting the pH to 6.8 with dilute HNO_3, then transferring the solution to a 100-mL volumetric flask and diluting to volume.

The phosphate concentration in solutions to be analyzed was determined by measurement of the phosphorus concentration of the sample by ICP-AES and assuming it all to be present as phosphate. With knowledge of the phosphate concentration the free sodium and potassium levels were calculated and these values then used for the determination of the free calcium and magnesium. Results for a set of analyses are shown in Table 4.6.

4.4.4 Determination of Free Calcium and Magnesium in a System Containing All Three Complexing Ligands: Citrate, Sulphate and Phosphate

This experiment was carried out in the same way as those outlined in the previous sections except that all the three ligands were combined in a single solution. The concentrations were selected to be similar to those found in normal urine samples, and so the system now approaches the level of a synthetic urine sample from the standpoint of complexing labile ligands [16]. Calculation of free ion concentrations was done using the computer program described in Appendix II. The process is somewhat more complicated for this multiple ligand system, but otherwise the treatment was as before. The results are shown in Table 4.7. Considering that only one experiment was run, and the uncertainties in the values calculated from the stability constants, the agreement can be considered to be quite satisfactory.

4.5 *Study of Potential Interference to the Method From Zinc(II) and Urea*

In section 4.4, the method under development was applied to systems containing three different complexing ligands at concentrations similar to those in urine. It was found that measured values were quite close to calculated free concentrations, thereby showing the method to be worth considering further for the measurement of free metal concentrations in urine. But before working on actual urine samples it was decided that other potentially interfering inorganic and organic species should be studied. One type of potential interference is from other divalent cations which could exchange onto the resin phase. The most likely problem ion of this kind is Zn^{2+}, the major heavy metal ion in urine. Another possible interferent, ammonium ion, is accounted for by being included in the standards for the calibration of the column. Another kind of interference might come from organic substances that adsorb on the resin. The most likely problem species of this type is urea, which is present in quite high concentrations (0.2 to 0.5 M) in urine. Accordingly, both zinc(II) and urea were investigated as possible interferences.

A 1000 ppm solution of $Zn(NO_3)_2$ was prepared by dissolving 0.4552 g of $Zn(NO_3)_2 \cdot 6H_2O$ (F.W. 297.47, Baker Analytical Reagent) in water and diluting to 100 mL with pure water. Urea (Analytical Reagent, BDH, Lot No.107471/28492) was used as the solid. Sample solutions were prepared by adding varying amounts of $Zn(NO_3)_2$ solution, urea, and fixed amounts of sodium, potassium, calcium and magnesium nitrate solutions into 25-mL volumetric flasks. The final concentrations of sodium, potassium, calcium and magnesium in each flask were 0.1 M, 0.04 M, 1.6 mM and 1.6 mM, respectively. The solutions were then analyzed using the method described in the previous section. The results are shown in Table 4.8.

It can be seen from Table 4.8 that in the range of normal urine samples, zinc and urea do not interfere with the measurement of free calcium and magnesium concentrations. The concentration of zinc present in urine samples appears to be sufficiently low that even though a small quantity likely sorbs onto the resin phase and displaces some of the four major ions, the level is too low to affect the results for free calcium and magnesium. The presence of a neutral species such as urea does not affect the column behavior even when present in concentrations a high as 0.5 M. It appears likely that the procedure would allow other ions at ppm levels, and other neutral organic molecules at quite high concentrations, to also be present without causing interference.

Table 4.4 Results for determination of free calcium and magnesium in solutions containing citrate. Concentrations are in mM.

Experimentally determined values			Calculated	Total
(based on Na calc.)	(based on K calc.)	Average	value from stability consts.	concn
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
3.6	3.2	3.4	3.5	4.8
1.5	1.4	1.5	1.6	3.2
2.4	2.1	2.3	2.2	3.2
2.6	2.2	2.4	2.5	4.8
3.9	3.6	3.8	3.8	4.8
1.4	1.3	1.4	1.6	3.2
2.5	2.4	2.4	2.4	3.2
2.9	2.8	2.8	2.9	4.8
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
3.0	2.7	2.9	3.0	4.0
2.9	2.6	2.8	3.2	6.0
3.1	2.7	2.9	2.9	4.0
2.2	1.9	2.0	2.2	4.0
5.0	4.6	4.8	4.8	6.0
1.8	1.6	1.7	2.0	4.0
4.8	4.6	4.7	4.7	6.0
3.7	3.5	3.6	3.8	6.0

Table 4.5 Results for determination of free calcium and magnesium in solutions containing sulphate. Concentrations are in mM.

Experimentally determined values			Calculated	Total
(based on Na calc.)	(based on K calc.)	Average	value from stability consts.	concn
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
4.0	3.9	4.0	3.5	4.8
1.9	1.8	1.9	1.8	3.2
2.6	2.6	2.6	2.5	3.2
3.3	3.1	3.2	3.0	4.8
4.3	4.0	4.2	3.8	4.8
2.6	2.3	2.5	2.1	3.2
2.9	2.7	2.8	2.6	3.2
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
3.2	3.2	3.2	3.1	4.0
3.7	3.5	3.6	3.7	6.0
3.3	3.3	3.3	3.2	4.0
2.8	2.7	2.7	2.7	4.0
5.3	5.0	5.2	5.0	6.0
3.1	2.8	2.9	2.8	4.0
5.5	5.3	5.4	5.1	6.0

Table 4.6 Results for determination of free calcium and magnesium in solutions containing phosphate. Concentrations are in mM.

Experimentally determined values			Calculated	Total
(based on Na calc.)	(based on K calc.)	Average	value from stability consts.	concn
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
4.7	5.2	4.9	4.7	4.8
2.5	3.1	2.8	2.8	3.2
3.0	3.4	3.2	3.1	3.2
4.3	4.7	4.5	4.5	4.8
4.3	4.8	4.6	4.7	4.8
2.9	3.3	3.1	3.0	3.2
2.7	3.0	2.9	2.6	3.2
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
3.8	4.2	4.0	3.7	4.0
4.4	5.5	4.9	4.8	6.0
3.8	4.3	4.1	3.7	4.0
3.6	3.9	3.8	3.5	4.0
5.6	6.2	5.9	5.6	6.0
3.6	4.1	3.9	3.5	4.0
4.6	5.3	4.9	4.7	6.0

Table 4.7 Results for determination of free calcium and magnesium in the presence of sulphate, phosphate and citrate as complexing ligands. All concentrations are in mM.

Experimentally determined values			Calculated	Total
(based on Na calc.)	(based on K calc.)	Average	value from stability consts.	concn
[Ca^{2+}]	[Ca^{2+}]	[Ca^{2+}]	[Ca^{2+}]	Ca
3.0	2.9	3.0	2.8	4.8
1.0	1.0	1.0	1.0	3.2
1.2	1.2	1.2	1.2	3.2
2.5	2.5	2.5	2.5	4.8
2.0	2.0	2.0	2.0	4.8
0.9	0.9	0.9	1.0	3.2
1.1	1.1	1.1	1.1	3.2
1.6	1.6	1.6	1.7	4.8
[Mg^{2+}]	[Mg^{2+}]	[Mg^{2+}]	[Mg^{2+}]	Mg
2.6	2.5	2.6	2.5	4.0
1.9	1.8	1.8	2.0	6.0
1.5	1.4	1.5	1.6	4.0
2.1	2.1	2.1	2.1	4.0
2.3	2.3	2.3	2.5	6.0
1.1	1.1	1.1	1.3	4.0
2.1	1.9	2.0	2.2	6.0
1.9	2.0	2.0	2.1	6.0

Table 4.8 Determination of free calcium and magnesium in the presence of zinc(II) and urea. All concentrations are in mM.

Sample no.	Species added	Experimentally determined values		
		(based on Na^+ calc.)	(based on K^+ calc.)	Average
		Ca^{2+}	Ca^{2+}	Ca^{2+}
1	--	1.5	1.6	1.6
2	1 ppm Zn^{2+}	1.5	1.6	1.5
3	2 ppm Zn^{2+}	1.5	1.6	1.5
4	10 ppm Zn^{2+}	1.5	1.6	1.6
5	0.1 M urea	1.6	1.6	1.6
6	0.3 M urea	1.6	1.6	1.6
7	0.5 M urea	1.7	1.6	1.7
		Mg^{2+}	Mg^{2+}	Mg^{2+}
1	--	1.6	1.7	1.6
2	1 ppm Zn^{2+}	1.5	1.6	1.6
3	2 ppm Zn^{2+}	1.5	1.6	1.6
4	10 ppm Zn^{2+}	1.5	1.6	1.6
5	0.1 M urea	1.6	1.6	1.6
6	0.3 M urea	1.6	1.6	1.6
7	0.5 M urea	1.7	1.6	1.7

4.6 *Analysis of a Urine Sample*

From previous results we see that free calcium and magnesium can be measured in the presence of citrate, sulphate and phosphate with the proposed method, and that zinc and urea at typical urine levels do not interfere with the measurement. Therefore it seems feasible for the method to be used for the analysis of a real urine specimen.

A urine sample was collected from a healthy volunteer, the pH recorded, and a portion of the sample immediately pumped through the column of Amberlyst resin used in the previous work. Unfortunately the column quickly became blocked, apparently by particulates that precipitated from the urine on cooling to room temperature. The flow rate became very slow and a high pressure buildup was observed in the tubing connecting the column to the pump.

A filter funnel with a 4 to 8 μm frit (ACE Glass, Vineland, N.J. USA) was therefore employed to filter the sample. Total concentrations of sodium, potassium, calcium, magnesium, sulphur and phosphorus were measured in the urine sample before and after filtration. It was found that filtration did not change these concentrations significantly.

A urine specimen after filtration was pumped through the column for about 30 min. to make sure that equilibrium was achieved between the resin and solution phases. The sodium, potassium, calcium and magnesium measurements in the resin phase were the same as described in the previous sections.

A separate aliquot of the urine was analyzed for total sulphur, phosphorous, sodium, potassium, calcium and magnesium by ICP-AES as follows: (1) 2 mL of urine sample was delivered into a 10-mL volumetric flask, followed by addition of 1 mL of 2 M HNO_3 and dilution to 10 mL; this solution A was used for the total determination of sulphur and phosphorous. (2) 2 mL of solution A was delivered into a 10-mL volumetric flask, followed by addition of 0.8 mL of 2 M HNO_3 and dilution to 10 mL. This solution B was used for total determination of calcium and magnesium. (3) 2 mL of solution B was delivered to a 10-mL volumetric flask, followed by addition of 0.8 mL of 2 M HNO_3 and dilution to 10 mL. This solution C was used for the determination of total sodium and potassium.

The free sodium and potassium ion concentrations were calculated from the ICP-determined values for totals of all the ions determined (all of the sulfur and phosphorus was assumed to be present as sulfate and phosphate) by computer calculation of the multiple equilibria involved. For this calculation the stability constants used were: for $NaSO_4^-$, log K = 0.68 ($\mu = 0$, 25°C); for KSO_4^-, log K = 0.79 (0, 25); for HSO_4^-, log K = 1.97 (0, 25); for $NaPO_4^{2-}$, log K = 0.75 (0.15, 37); for $NaHPO_4^-$, log K = 0.60 (0.2, 25); for NaH_2PO_4, log K = 0.114 (0.3, 37); for KPO_4^{2-}, log K = 0.60 (0.15, 37); for $KHPO_4^-$, log K = 0.48 (0.15, 37); for KH_2PO_4, log K = -0.20 (0.3, 37); for HPO_4^{2-}, log K = 12.35 (0, 25); for $H_2PO_4^-$, log K= 7.199 (0, 25); for H_3PO_4, log K = 2.148 (0, 25). For different urine samples, the ionic strength was taken as the total sodium and potassium concentrations, and all constants were adjusted to the ionic strength of each sample.

Mixed constants were calculated from calibration results for the column and the amount of metal ions found in the resin phase. The ionic strength of the solution was estimated from the total sodium and potassium concentrations, and was used to calculate activity coefficients in the solution phase. The free calcium and magnesium concentrations were then calculated using the concentration constants, the analyses of the metal ion concentrations sorbed on the resin phase, and the free sodium and potassium concentrations in the solution phase. A computer program was written in BASIC to carry out these calculations; it is listed in Appendix III.

Table 4.9 shows the results of the free calcium and magnesium measurements in this urine sample. Since no reliable independent method is available, it is not possible to

Table 4.9 Determination of free calcium and magnesium in a normal urine sample using Amberlyst 15 resin. pH of sample was 5.4.

	Total conc. found	Free Ion Concentration from Ion-Exchange		
		calc'd fm Na conc.	calc'd fm K conc.	Average
Calcium	3.36 mM	2.25 mM	2.02 mM	2.11 mM (63%)[a]
		2.25 mM	1.90 mM	
Magnesium	2.14 mM	1.34 mM	1.20 mM	1.26 mM (59%)[a]
		1.35 mM	1.14 mM	
Sodium	0.224 M			0.215 M[b]
Potassium	0.066 M			0.064 M[b]

[a] Values in parentheses are percentages of the total concentrations present in the free ion form.

[b] Free ion concentrations calculated from their total concentrations, corrected for complexation by sulphate and phosphate at the urine pH.

verify the values obtained. However, the fractions of free calcium and magnesium found fall within the range estimated by medical workers [22-27].

The problem of blockage of the column by urine may be caused by plugging of the frits used to hold the resin in the column, or by plugging of the pores of the resin itself. In either case, prefiltration of the urine appears to solve the problem.

4.7 *Conclusions*

In this chapter it was shown that free calcium and magnesium in solution can be determined by the ion-exchange equilibration technique using an Amberlyst 15 resin column and an ARL 15000 direct reader ICP-AES as detector. The direct reader spectrometer allowed the simultaneous determination of sodium and potassium as well as calcium and magnesium on the resin. It also allowed determination of the amount of sulphur and phosphorous in samples so that complexation of sodium and potassium by these ions could be taken into account. The method gave satisfactory results in solutions containing ligands such as citrate, sulphate, and phosphate, as well as zinc(II) and urea, all at concentrations typically found in urine. The major problem encountered for practical application was blockage of the column by particulate matter in urine. To avoid this problem it was decided to investigate cation exchange resins other than the macroporous material used here, and possibly to consider use of a smaller column. The next chapter describes work in this area.

Chapter 5 Study of Dowex 50W x2 Resin for Free Metal Ion Speciation by the Ion-Exchange Equilibration Method

5.1 *Introduction*

In the previous chapters, Amberlyst 15 resin was used to construct a column for the determination of free calcium and magnesium in urine by the ion-exchange equilibrium method. Because a fairly large quantity of resin was used in the column (25 mg), equilibrium times were long (≈15 minutes). Also, before urine samples were pumped through the column it was necessary to pass them through a filter to get rid of particulate matter, otherwise the column was susceptible to plugging. Even with filtration, flow rates tended to be low, and backpressure in the system unacceptably high. Therefore other resins were investigated to determine whether replacement of the resin or modification of the column design would make handling of real urine samples easier.

It was also felt to be important to explore other resins to see whether they could be applied to the ion-exchange equilibration method in the same way as Amberlyst 15 was in the previous chapters. For these reasons two additional strongly acidic cation exchange resins, Dowex 50W x2 and Dowex 50W x8, were investigated. In this chapter, the behavior of a column containing Dowex 50W x2 resin is discussed. It was thought that the low degree of cross-linking in this resin (2%) might make solute contact with the exchange sites more rapid, thus speeding up equilibration. Also, plugging of the resin by particulates might be less of a problem.

As an additional study, both nitrate and chloride salts of the major cations were used to calibrate the column. Any differences seen in the selectivity constants of the ion exchange equilibria when the anion was changed might be due to different ion-pair affinities of chloride and nitrate anions with sodium, potassium, calcium, and magnesium. Since in urine samples the chloride ion concentration is close to that of the sodium ion, while the nitrate ion concentration is negligible, it was thought to be better to evaluate the use of chloride salts to calibrate the column for urine analyses.

5.2 *Experimental Section*

5.2.1 Column Construction and Chemicals

A column containing 5.5 mg of 200 to 325 mesh Dowex 50W x2 resin was constructed using the technique described in Chapter 3. The resin was from a batch that had been previously cleaned and de-fined by A. Hewavitharana in this laboratory [16].

A 1 M solution of sodium chloride was prepared by dissolving 29.2198 g of analytical reagent grade NaCl (F.W. 58.44, BDH Lot 112968-20432, Toronto) in water and diluting to 500 ml. Similarly, a 0.5 M solution of potassium chloride was prepared by dissolving 18.6373 g of analytical reagent grade KCl (F.W. 74.55, BDH Lot 107975-31071, Toronto) in water and diluting to 500 ml. Similarly, a 0.08722 M solution of calcium chloride was prepared by dissolving 5.5510 g of reagent grade $CaCl_2$ (F.W. 110.99, Anachemia, Lot 091205, Montreal/Toronto) in water and diluting to 500 ml. This solution was standardized by titration with EDTA using calmagite as indicator. On the basis of the anhydrous salt a purity of 87.2% was found. Because of the hydroscopicity of this material it is probable that the remaining 13% is water; this percentage corresponds to approximately a monohydrate.

A 0.1005 M solution of magnesium chloride was prepared by dissolving 10.1650 g of $MgCl_2{\cdot}6H_2O$ (F.W. 203.30, Anachemia Lot 781125, Montreal/Toronto) in water and diluting to 500 ml. This solution was also titrated with EDTA using calmagite as indicator; a purity of 100.5% was found. A 1 M solution of ammonium chloride was prepared by weighing 5.349 g of reagent grade NH_4Cl (F.W. 53.49, Anachemia, Montreal/Toronto), dissolving in water and diluting to 100 ml. The solutions of nitrate salts used were those described previously in Chapter 3.

5.2.2 Experimental Procedure

The same experimental procedures was used as in the previous chapters except that the new column containing Dowex 50 x2 described above replaced the earlier column of Amberlyst 15. Also, the eluted solutions were collected in 10-ml instead of 25-ml volumetric flasks because the elution volumes from the smaller column were correspondingly smaller.

5.3 *Results and Discussion*

5.3.1 Equilibrium and Elution Times

A single standard solution was prepared for the equilibrium and elution time studies with the chloride anion. The composition of this standard, which was chosen to provide levels slightly less than the normal levels reported for these salts, was: $CaCl_2$, 1.74 mM; $MgCl_2$, 2.00 mM; NaCl, 0.1 M; KCl, 0.05 M. For the equilibrium time study, 2 M nitric acid was used as eluent and the elution time was fixed at 2 minutes; the standard solution was passed through the column for 1, 2, 5, 10, and 20 minutes prior to washing and elution. For the elution time study, with 2 M nitric acid as eluent and a fixed equilibration time of 3 minutes, the elution time was varied from 20 seconds to 2.5 minutes (20, 40, 60, 90, 120, and 150 seconds). The results, shown in Figure 5.1, indicate that 2 minutes is sufficient for equilibration and that 20 seconds is sufficient to elute all of the sorbed metal ions from the column. In all succeeding studies 5 minutes was used for column

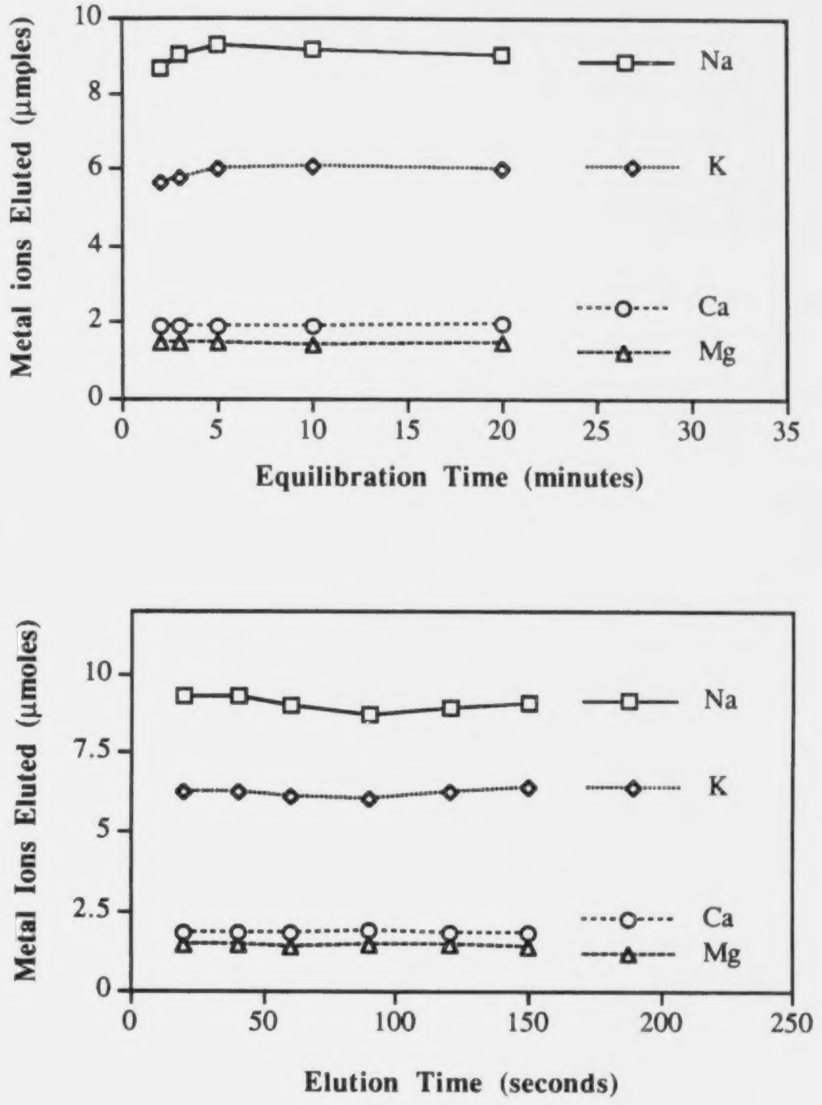


Figure 5.1. Results of study of equilibrium and elution times for sodium, potassium, calcium, and magnesium from 5.5-mg Dowex 50W x2 resin column.

equilibration and 1 minute for column elution. These times were thus set at two and a half to three times the minimum to provide a margin of safety.

5.3.2 Calibration of Dowex 50W x2 Column Using Chloride Salts of the Metal Ions as Standards

Chloride, not nitrate, is the major anion present in urine. Because chloride may be expected to have different solution properties than nitrate, such as the capability of ion pairing, it was decided to use chloride salts to prepare standards for calibration of the column. A set of standards were also prepared from nitrate salts for comparison. The compositions of the standard solutions are shown in Table 5.1. Twenty standards were prepared, 3 of them containing varying amounts of NH_4Cl so that the interference of ammonium ion could be eliminated, as discussed previously.

Each standard was passed through the column for 5 minutes to achieve equilibrium between the solution and the resin phases, followed by passage of air through the column for 1 minute, then water for 1 minute. The metal ions sorbed on the column were then eluted by passage of 2 M HNO_3 for 1 minute. The eluent, along with a subsequent 1-minute water wash of the column, was collected in a 10-ml volumetric flask. The solution in the flask was diluted to mark with water, then analyzed on an ARL 34000 ICP/AES instrument for sodium, potassium, calcium and magnesium to yield the metal ion concentrations in the resin phase. For convenience the units used for this determination were micromoles/column; this is convenient so long as the same column is used. With knowledge of the free metal ion concentrations (here the free metal ion concentration was the same as the total metal ion concentration), the previously defined mixed selectivity constants for the four ion combinations of Ca/Na, Ca/K, Mg/Na and Mg/K could be calculated.

Table 5.1 Composition of standards prepared from chloride salts for calibration of Dowex 50W x2 resin column. All concentrations are in mM.

No.	[NaCl]	[KCl]	[$CaCl_2$]	[$MgCl_2$]	[NH_4Cl]
1	60	20	0.7	0.8	0
2	60	40	1.4	1.6	0
3	60	80	2.8	4.0	0
4	60	120	4.2	6.0	0
5	100	20	1.4	4.0	0
6	100	40	0.7	6.0	0
7	100	80	4.2	0.8	0
8	100	120	2.8	1.6	0
9	200	20	2.8	6.0	0
10	200	40	4.2	4.0	0
11	200	80	0.7	1.6	0
12	200	120	1.4	0.8	0
13	280	20	4.2	1.6	0
14	280	40	2.8	0.8	0
15	280	80	1.4	6.0	0
16	280	120	0.7	4.0	0
17	200	80	2.8	4.0	0
18	100	40	1.4	1.6	20
19	100	40	1.4	1.6	50
20	100	40	1.4	1.6	100

For the Amberlyst 15 resin it was shown that the log value of the mixed constant could be related to the metal ion concentrations in the resin phase. To test whether the same held true for the Dowex 50W x2 resin, a similar calculation was carried out. It was found that the percent difference between the experimental and predicted values was relatively large, and that the coefficient of correlation between them was small. This means that the metal ion composition in the resin phase itself is not sufficient to account for the variation in the selectivity constant values, and that other factors must be included if we are to be able to predict these values with any precision and accuracy.

For Dowex 50W x2 resin, with a crosslinking of 2%, swelling can be expected to be relatively large compared to similar resins of greater crosslinking. Since swelling of the resin is mainly determined by the resin crosslinking and the ionic strength of the solution phase, the ionic strength of the solution should also be taken into account as an additional factor when predicting mixed selectivity constant values. Several functions of ionic strength, including the inverse and the square root, were therefore incorporated in the calculation of the mixed constants. It was found empirically that the square root of ionic strength gave the best results. Therefore, for Dowex 50W x2 resin the log values of mixed selectivity constants were calculated by a function involving the fractions of the four metal ions present in the resin phase and the square root of the ionic strength of the solution phase. The results are shown in Tables 5.2a to 5.2d in the columns headed (F5), together with predicted values in the columns headed (F4). The fraction of metal ions in the resin phase was used here for comparison. In these tables the relative standard error of estimation (RSEE) is defined as [143]

$$\mathrm{RSEE} = \frac{\sqrt{\dfrac{\Sigma\,(\hat{Y}_i - Y_i)^2}{n-2}}}{\bar{Y}} \times 100\% \qquad (1)$$

where $\widehat{Y}_i$, Y_i and $\overline{Y}$ are the predicted, experimental, and average of the experimental values of the mixed selectivity constants, respectively, and n is the number of standard solutions. Also, the coefficient of correlation (R), as

$$R = \frac{\Sigma Y_i \cdot \widehat{Y}_i - \frac{\Sigma Y_i \cdot \Sigma \widehat{Y}_i}{n}}{\sqrt{(\Sigma Y_i^2 - \frac{(\Sigma Y_i)^2}{n}) \cdot (\Sigma \widehat{Y}_i^2 - \frac{(\Sigma \widehat{Y}_i)^2}{n})}} \qquad (2)$$

Smaller RSEE values and larger R values indicate that the predicted values are closer to the experimental values, and better regression results have been obtained. Tables 5.2a to 5.2d show that all the RSEE values are smaller, and the R values larger, for calibrations that consider the effect of ionic strength of the solution phase. Therefore the ionic strength effects turned out to be important for Dowex 50W x2 resin where the crosslinking is low, and need to be taken into account when estimating the mixed selectivity constants.

5.3.3 Calibration of Dowex 50W x2 Column Using Nitrate Salts for Preparation of Standards

As mentioned, in urine samples chloride is the major anion present, and since chloride is expected to have different solute properties compared to nitrate ion, it seems better to use chloride salts when preparing standards for calibration of the ion-exchange column when the results are going to be used for the analysis of urine samples. Of interest, however, is the extent of the difference in behavior of the two ions under similar conditions, especially with respect to ion-pairing of chloride with the cations used in the

Table 5.2a Comparison of experimental values for $K^{mix}_{Ca/Na}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5).

Standard Number	Experimental	Calculated (F4)	Calculated (F5)
1	239	272	244
2	254	285	272
3	300	293	301
4	335	296	317
5	291	273	273
6	266	270	266
7	290	281	288
8	282	273	272
9	258	254	263
10	249	259	270
11	235	240	224
12	237	246	238
13	251	239	247
14	255	234	234
15	258	241	252
16	224	240	249
17	255	253	259
18	244	258	244
19	240	252	246
20	251	245	252
RSEE		6.9%	4.8%
R		0.754	0.889

Table 5.2b Comparison of experimental values for $K^{mix}_{Ca/K}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5).

Std. No.	Experimental	Calculated F4	Calculated F5
1	124	138	125
2	129	143	138
3	151	149	152
4	169	152	162
5	145	134	133
6	136	136	134
7	153	148	151
8	160	152	151
9	126	130	134
10	123	133	138
11	133	138	130
12	137	143	139
13	138	128	131
14	142	130	130
15	144	134	140
16	128	138	143
17	145	138	141
18	131	140	134
19	134	142	139
20	148	144	147
RSEE		7.0%	5.7%
R		0.622	0.773

Table 5.2c Comparison of experimental values for $K^{mix}_{Mg/Na}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5).

Std. No.	Experimental	Calculated F4	Calculated F5
1	157	181	158
2	168	188	178
3	198	195	201
4	222	198	215
5	187	179	178
6	181	182	179
7	208	189	194
8	193	192	191
9	176	171	179
10	169	175	184
11	174	176	162
12	163	181	175
13	176	165	172
14	171	166	166
15	185	173	183
16	186	176	185
17	176	178	183
18	169	178	167
19	170	176	171
20	176	173	179
RSEE		7.1%	4.3%
R		0.611	0.879

Table 5.2d Comparison of experimental values for $K^{mix}_{Mg/K}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5).

Std. No.	Experimental	Calculated F4	Calculated F5
1	81	91	81
2	85	95	90
3	99	97	102
4	112	102	110
5	93	88	87
6	93	92	90
7	110	99	102
8	109	107	106
9	85	87	91
10	83	90	94
11	98	101	94
12	94	105	102
13	97	88	91
14	95	93	93
15	103	96	101
16	106	102	106
17	100	97	100
18	91	97	91
19	95	99	96
20	103	102	105
RSEE		7.1%	5.0%
R		0.660	0.847

system. Therefore a set of standards was prepared from nitrate salts and run under the same conditions as chloride to permit comparison of the two systems. It was also of interest to explore further the effects of increased resin-swelling effects on selectivity constants obtained for the Dowex 50W x2 resin.

A set of 20 standards was prepared, 3 of the standards containing differing amounts of NH_4NO_3 so that the interference from ammonium ion could be eliminated. The compositions of the standards are shown in Table 5.3. These standards were run through the column and analyzed by the procedure for the calibration using chloride salts outlined in the previous section. Calculations were carried out as for the chloride system; the results are shown in Tables 5.4a to 5.4d. The uncertainties in the experimental values in these tables are estimated to be of the order of 5% relative. This quantity is based on a propagation of error analysis using 2% relative as the estimated uncertainty in the ICP measurements.

From the results shown in these tables it can be seen that the predicted values are much closer to the experimental values when the ionic strength is included in the calibration expressions.

5.3.4 Comparison of Selectivity Constants Obtained Using Chloride and Nitrate Salts

Strong electrolytes such as NaCl dissociate essentially completely in aqueous solutions to produce the hydrated ions. When the concentration of a strong electrolyte is very low, interaction between the hydrated ions is negligible, the activity coefficient of the ions can be considered equal to 1, and the activity of the ion considered to be the same as its concentration. As the concentration of the electrolyte is gradually increased but still low, interaction between the ions is no longer negligible, and the activity coefficient of the ion becomes less than unity. Its value may be estimated from the Debye-Huckel limiting law

Table 5.3. Composition of standards for calibration of Dowex 50W x2 resin column using nitrate salts. All concentrations are given in mM.

No.	$[NaNO_3]$	$[KNO_3]$	$[Ca(NO_3)_2]$	$[Mg(NO_3)_2]$	$[NH_4NO_3]$
1	60	20	0.8	0.8	0
2	60	40	1.6	1.6	0
3	60	80	3.2	4.0	0
4	60	120	4.8	6.0	0
5	100	20	1.6	4.0	0
6	100	40	0.8	6.0	0
7	100	80	4.8	0.8	0
8	100	120	3.2	1.6	0
9	200	20	3.2	6.0	0
10	200	40	4.8	4.0	0
11	200	80	0.8	1.6	0
12	200	120	1.6	0.8	0
13	300	20	4.8	1.6	0
14	300	40	3.2	0.8	0
15	300	80	1.6	6.0	0
16	300	120	0.8	4.0	0
17	200	80	3.2	4.0	0
18	100	40	1.6	1.6	20
19	100	40	1.6	1.6	50
20	100	40	1.6	1.6	100

Table 5.4a Comparison of experimental values for $K^{mix}{}_{Ca/Na}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5) for nitrate system.

Std. No.	Experimental	Calculated F4	Calculated F5
1	189	248	200
2	224	259	235
3	278	271	284
4	315	277	316
5	255	254	248
6	276	271	264
7	268	253	248
8	288	272	272
9	248	243	257
10	265	241	264
11	227	258	227
12	241	264	249
13	240	229	241
14	255	235	237
15	262	253	276
16	284	260	282
17	260	256	268
18	263	256	236
19	239	258	246
20	267	262	273
RSEE		9.3%	4.3%
R		0.498	0.914

Table 5.4b Comparison of experimental values for $K^{mix}_{Ca/K}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5) for nitrate system.

Std. No.	Experimental	Calculated F4	Calculated F5
1	114	144	115
2	126	147	133
3	156	152	159
4	177	156	178
5	137	140	136
6	153	151	147
7	157	149	156
8	176	168	168
9	148	141	150
10	158	143	156
11	148	172	151
12	164	177	167
13	152	145	153
14	161	154	155
15	169	163	178
16	190	172	187
17	165	158	166
18	154	152	141
19	144	156	148
20	163	158	165
RSEE		9.2%	3.5%
R		0.604	0.954

Table 5.4c Comparison of experimental values for $K^{mix}{}_{Mg/Na}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5) for nitrate system.

Std. No.	Experimental	Calculated F4	Calculated F5
1	137	193	147
2	166	197	175
3	205	200	216
4	233	203	239
5	185	185	180
6	196	188	182
7	235	105	217
8	224	212	212
9	193	186	200
10	205	192	215
11	183	206	175
12	182	213	197
13	207	193	206
14	213	198	199
15	211	198	221
16	228	205	227
17	209	200	212
18	202	202	183
19	188	206	194
20	218	212	223
RSEE		11%	5.4%
R		0.344	0.895

Table 5.4d Comparison of experimental values for $K^{mix}_{Mg/K}$ with values calculated from NaR, KR, CaR_2 and MgR_2 (F4), and from NaR, KR, CaR_2, MgR_2 and $\sqrt{\mu}$ (SQR(IS)) (F5) for nitrate system.

Std. No.	Experimental	Calculated F4	Calculated F5
1	82	112	85
2	93	112	99
3	115	112	118
4	131	114	134
5	100	102	99
6	108	105	101
7	137	121	128
8	137	131	131
9	115	108	117
10	123	114	127
11	120	137	117
12	124	143	132
13	131	122	130
14	135	129	130
15	136	127	142
16	153	136	151
17	132	124	131
18	118	120	109
19	113	124	117
20	133	128	135
RSEE		11%	4.4%
R		0.624	0.952

up to concentrations of the order of millimolar or so. When the concentration of the electrolyte becomes still higher the distance between the hydrated ions is reduced to a point where interactions between the ions begin to become significant. The extent of these interactions depends upon the individual ions. The Debye-Huckel equation has been extended and modified by many workers to take into account the size of the hydrated ions; with these modifications the equation can be used to calculate activity coefficients of ions with moderate accuracy up to concentrations of about 0.1 M. At higher concentrations the properties of the aqueous solution become increasingly different from those of dilute solutions. The activity of the water medium is reduced, along with its dielectric constant, and interactions between ions become even more complicated. Under these conditions activity coefficients of ions are best estimated by the Davies equation, an empirical expression that is nonetheless useful for most strong electrolytes at concentrations ranging up to 0.5 M or so [137].

The ionic strength of most urines lies in the range of 0.1 to 0.3. For solutions in this concentration range the Davies equation should be used to calculate activity coefficients of the ions. In the analytical method under study here ion pairing by chloride, if present, should be revealed by comparison of the calibration results for the chloride and nitrate systems. The average values of the selectivity constants should be the same for the two systems if no ion pairs are present because the two sets of standard solutions have similar cation concentrations. Table 5.5 shows that the average values of the selectivity constants (using 20 standards in each set) with chloride and nitrate salts are indeed different from each other. This suggests that ion pairing is occurring, and that the ion-pair affinities of chloride and nitrate with the four major cations are different. Further investigation of the ion-pair properties of chloride and nitrate with the four major cations of interest here were not made because literature values for the relevant ion pair constants vary over a wide range; also no literature value is available for the magnesium-nitrate ion pair. Since in urine samples the chloride ion concentration is close to that of the sodium ion, while the nitrate ion concentration is negligible, it appears to be better to use chloride salts for calibration of the column when urine samples are to be analyzed.

For the determination of free calcium and magnesium in urine, ion pairing can be ignored in the calculations so long as the standards have similar compositions to the samples. Free metal ion concentrations obtained under these conditions, however, will include any ion pairs present in the standards and samples. This was not considered to be a problem because of the small size of the ion pair association constants encountered in this work, and the lability of the ion pairs. Free metal ion concentrations measured in this way can still give useful information for diagnostic purposes. It should be recognized, however, that when chloride salts are used in the preparation of standards for calibration of a resin column, ion pairing by chloride to the metals will occur to some extent. Under these conditions the values for the free calcium and magnesium concentrations that are obtained by the method will include the $M^{n+}Cl^-$ ion-pair species.

5.3.5 Application of Dowex 50W x2 Resin to the Determination of Free Calcium and Magnesium Concentrations in the Presence of Citrate as Complexing Ligand

After the Dowex 50W x2 column was calibrated, a coefficient matrix was obtained and solutions containing citrate ion as complexing ligand were analyzed to compare calculated and measured free calcium and magnesium values in the same way as was done in the previous chapter with Amberlyst resin. For this purpose a series of sample solutions were prepared in 25-ml volumetric flasks, each containing varying amounts of the chloride salts of sodium, potassium, calcium, and magnesium, along with potassium citrate as complexing ligand. This was followed by measurement of the pH as before. The composition and pH of each solution is listed in Table 5.6. The solutions were then passed

Table 5.5 Comparison of averages of selectivity constants calculated using chloride and nitrate salts in the calibration of a column containing Dowex 50W x2 resin. The uncertainties in these values are estimated to be of the order of 5 to 10% relative.

K expression	Chloride salts	Nitrate salts
$K_{Ca/Mg}$	1.45	1.28
$K_{K/Na}$	1.37	1.29
$K^{mix}_{Ca/Na}$	261	257
$K^{mix}_{Ca/K}$	140	156
$K^{mix}_{Mg/Na}$	180	201
$K^{mix}_{Mg/K}$	97	122

Table 5.6 Composition and pH values of solutions prepared for a study of the citrate system. All concentrations are given in mM.

No.	NaCl	KCl	$CaCl_2$	$MgCl_2$	$K_3Citrate$	pH
1	100	100	2.8	4.0	3.0	4.95
2	100	100	4.2	4.0	6.0	4.97
3	200	40	4.2	6.0	3.0	4.82
4	200	40	2.8	4.0	6.0	5.01
5	200	100	2.8	6.0	3.0	4.86
6	200	100	4.2	6.0	6.0	4.88

through the calibrated resin column, the column washed, and the sorbed ions eluted with 2 M HNO_3 into 10-ml volumetric flasks. The flask contents were diluted to volume with water and analyzed by ICP-AES on an ARL 34000 direct reader spectrometer. From the stability constants of sodium and potassium with citrate it was calculated that 99.7% or more of these cations could be considered to be in the free, uncomplexed form under the conditions of the experiment, and so the free sodium and potassium concentrations were taken as equal to their total concentrations.

With knowledge of (1) the free sodium and potassium ion concentrations in each solution, (2) the metal ion concentrations in the resin phase, and (3) the coefficient matrix obtained from the set of standards, it was possible to calculate the concentrations of free calcium and magnesium in the citrate solutions. These values were then compared with values calculated from the total ligand and total metal ion concentrations, the stability constants for all of the complexes present, and the pH. The results are shown in Table 5.7. Here again the uncertainties in the experimental averages are estimated to be of the order of 10% relative.

From Table 5.7 we see that the experimentally determined free calcium and magnesium concentrations are in reasonable agreement with calculated values. This provides additional confirmation that the method selectively measures free calcium and magnesium concentrations in the presence of kinetically labile ligands such as citrate. It also shows that Dowex 50W x2 resin can be used in place of Amberlyst 15 as the ion exchanger.

Table 5.7 Results for determination of free calcium and magnesium in the presence of citrate as complexing ligand, using Dowex 50W x2 resin. All concentrations given in mM.

Experimentally determined values			Calculated values from stability consts.	Total conc.
(based on Na calc.)	(based on K calc.)	Average		
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
2.0	2.2	2.1	2.1	2.8
2.4	2.7	2.5	2.6	4.2
3.4	3.7	3.6	3.5	4.2
1.8	1.9	1.9	1.7	2.8
2.4	2.7	2.6	2.3	2.8
3.0	3.4	3.2	2.9	4.2
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
2.7	3.1	2.9	3.2	4.0
2.2	2.6	2.4	2.7	4.0
4.8	5.2	5.0	5.2	6.0
2.6	2.7	2.7	2.6	4.0
5.0	5.6	5.3	5.1	6.0
4.3	4.9	4.6	4.4	6.0

5.4 *Conclusions*

In this chapter, Dowex 50W x2 resin was employed for the determination of free calcium and magnesium in solution by the ion-exchange equilibration technique. Both chloride and nitrate salts were used to prepare standards for calibration of the column. Ion pairing of chloride and nitrate with calcium and magnesium was shown to be present by comparing values of selectivity constants obtained for the two sets of standards. It is possible to minimize the effect of ion pairing on estimation of free metal ion measurements in solutions containing chloride by making the composition of the standards close to that of the samples. Thus, since the major anion in urine is chloride, chloride salts should be used in preparation of standards for urine analyses. The method was applied to a trial system containing citrate as complexing ligand, with satisfactory results.

It was shown that swelling by Dowex 50W x2 as a result of changes in the ionic strength of solutions coming in contact with it is relatively large. This made necessary the inclusion of ionic strength of the equilibrating solution as an additional factor in the expression used to predict selectivity constants of the resin for ions. Other resins with less swelling, such as Dowex 50W x8, warrant study to see whether it may be possible to obtain selectivity constants without incorporation of this additional factor. Such a study is described in the next chapter. Evaluation of the resin with a real urine sample was postponed until the question of the effects of resin swelling could be answered by comparison with the 50W x8 analog.

Chapter 6 Study of Dowex 50W x8 Resin for Free Metal Ion Speciation by the Ion-Exchange Equilibration Method

6.1 *Introduction*

In the previous chapters, two ion-exchange resins, Amberlyst 15 and Dowex 50W x2, were studied as sorbents for the determination of free calcium and magnesium by the equilibration method. For Amberlyst 15 resin, blocking of the column by particles in urine samples happened, probably due to the small size of the pores in the structure of the resin beads. This discouraged its application for this work. As for Dowex 50W x2 resin, its swelling properties make it difficult to predict resin selectivity because the ionic strength of the solution becomes a significant factor. It is therefore useful to study new resins that have the desired properties for the determination of free ion concentrations in urine. Dowex 50W x8 resin, which has higher cross-linking and thus less swelling than Dowex 50W x2, as well as being more porous than Amberlyst 15, appeared to be the best candidate for our purpose. Preliminary experiments confirmed that Dowex 50W x8 was the best of the resins studied. Unlike Dowex 50W x2 resin, the ionic strength of the solution turned out not to be a significant factor in predicting mixed selectivity constants. This simplified the calculations. Also, analyses of several urine samples with this resin showed no plugging of the column by particulate matter, as was observed with Amberlyst 15.

Both chloride and nitrate salts of the four major cations present in urine were used to calibrate the column. The ion-pairing by chloride that was seen with Dowex 50W x2 was also observed with Dowex 50W x8. Since in urine samples chloride rather than nitrate is the major anion present, the calibration results for chloride were used for the urine sample analyses. A standard reference material (NIST SRM 2670, toxic ions in freeze dried urine) was analyzed to give a point of reference for the method even though the material is not certified for free calcium or magnesium.

6.2 *Experimental Section*

6.2.1 Column Construction and Chemicals

A column was constructed containing 6.4 mg of Dowex 50W x8 (200-400 mesh) resin in the same way as previously described in Chapter 5. The resin was from a batch that had been previously cleaned and de-fined by A. Hewavitharana in this laboratory [16]. The chloride and nitrate salt solutions were the same as in the previous chapters. All urine samples were freshly collected from healthy volunteers and used within one hour.

The standard reference material urine portions (SRM 2670, U.S. National Institute for Standards and Technology, Gaithersburg, MD) were reconstructed according to the instructions provided by adding 20.0 ml of pure water to each bottle and using the solution within an hour of preparation.

6.2.2 Experimental Method

The experimental procedure was the same as described in chapter 5.

6.3 *Results and Discussion*

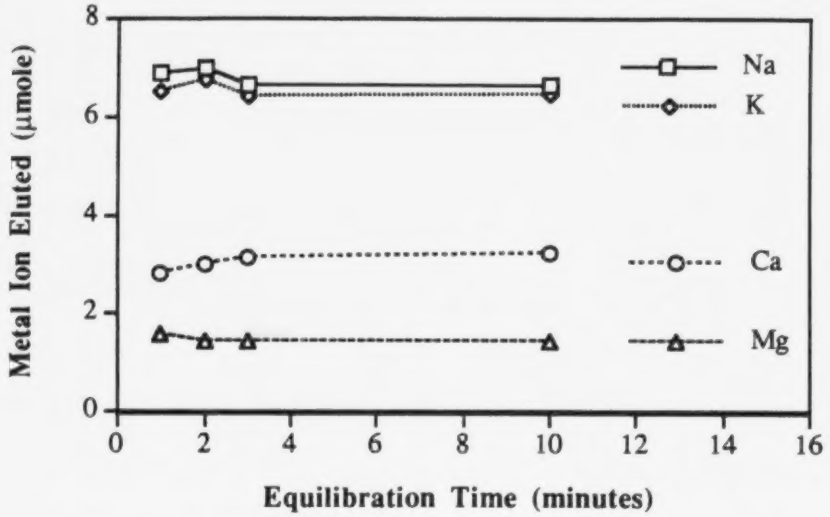
6.3.1 Establishment of Equilibrium and Elution Times

A single standard solution was prepared as before for the equilibrium and elution time studies with chloride salts. The composition, which was chosen to provide levels slightly less than the normal values reported for these salts in urine, was: $CaCl_2$, 1.74 mM; $MgCl_2$, 2.00 mM; NaCl, 0.1 M; and KCl, 0.05 M. For the equilibrium time study, with 2 M nitric acid as eluent and a fixed elution time of 1 minute, the solution was passed through the column for 1, 2, 3, and 10 minutes prior to washing and elution. For the elution time study, with 2 M nitric acid as eluent and a fixed column equilibration time of 3 minutes, the elution time was varied from 20 seconds to 100 seconds (20, 40, 60 and 100 seconds). The results, shown in Figure 6.1, indicate that 3 minutes is sufficient for equilibration and that 40 seconds is sufficient to elute all of the sorbed metal ions from the column. In all succeeding studies 5 minutes was used for column equilibration and 1 minute for column elution.

6.3.2 Calibration of the Dowex 50W x8 Column Using Chloride Salt Standards

The standards used to calibrate the Dowex 50W x8 resin column had exactly the same compositions as those used to calibrate the Dowex 50W x2 resin column, as was shown in Table 5.1. Each standard was analyzed in the same way as outlined for the Dowex 50W x2 column.

In contrast to results obtained with the Dowex 50W x2 resin, log values of the mixed selectivity constants for Dowex 50W x8 could be related to the composition of the metal ions in the resin phase without a term to take swelling of the resin into account. The results predicted from the composition of the four metal ions in the resin phase are shown in Tables 6.1a and 6.1b. Uncertainties in the experimental values are estimated to be in the order of 5 to 10% relative. These results suggest that the swelling effect of Dowex 50W x8 resin is not great enough to affect the selectivity of the resin over the ionic strength range encountered in normal urine samples.



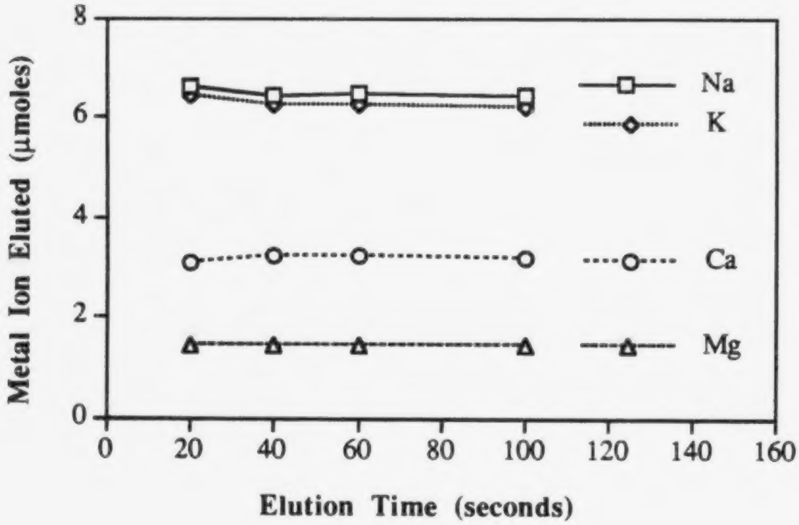


Figure 6.1 Equilibrium and elution time studies for Dowex 50W x8 resin column.

Table 6.1a Comparison of experimental and predicted K^{mix} values for calcium equilibria in chloride system using Dowex 50W x8 resin.

Solution number	$K^{mix}_{Ca/Na}$		$K^{mix}_{Ca/K}$	
	Expt.	Pred.	Expt.	Pred.
1	636	699	153	170
2	731	753	181	191
3	803	801	224	216
4	859	827	250	237
5	728	695	162	153
6	721	734	181	189
7	761	747	207	207
8	784	780	260	260
9	620	622	145	144
10	662	645	170	158
11	647	646	228	227
12	653	682	245	255
13	553	564	135	141
14	570	575	173	164
15	627	629	200	199
16	656	650	238	236
17	702	682	206	206
18	706	709	197	199
19	734	720	211	211
20	729	739	234	233
RSEE		3.3%		3.9%
R		0.955		0.978

Table 6.1b Comparison of experimental and predicted K^{mix} values for magnesium equilibria in chloride system with Dowex 50W x8 resin.

Solution number	$K^{mix}_{Mg/Na}$		$K^{mix}_{Mg/K}$	
	Expt.	Pred.	Expt.	Pred.
1	256	277	62	67
2	282	296	70	75
3	307	309	86	83
4	333	319	97	91
5	269	260	60	57
6	267	260	67	67
7	321	310	87	86
8	312	313	104	104
9	237	241	55	56
10	263	260	67	64
11	250	256	88	90
12	274	274	103	103
13	232	235	57	59
14	256	238	74	68
15	241	245	77	77
16	251	255	91	92
17	272	270	80	82
18	281	283	79	79
19	290	289	83	85
20	297	298	95	94
RSEE		3.3%		3.9%
R		0.950		0.979

6.3.3 Calibration of the Dowex 50W x8 Column Using Nitrate Salt Standards

Results reported in the previous chapter for Dowex 50W x2 resin showed that the average selectivity constants prepared with chloride salts were different from standards prepared with nitrate salts owing to differing ion-pair capabilities of the two anions. It was therefore of interest to see whether the same was true for the Dowex 50W x8 resin. Accordingly, the 50W x8 resin column was calibrated again, this time with solutions prepared from the corresponding nitrate salts. These standards had exactly the same compositions as those used to calibrate the 50W x2 resin column (Table 5.3), and each was analyzed in the same way as for that column.

Unlike the results obtained with Dowex 50W x2, log values of the mixed selectivity constants for Dowex 50W x8 could be related to the composition of the metal ions in the resin phase in the same way as for Amberlyst 15. Values predicted from the amounts of the four metal ions found in the resin phase are shown in Tables 6.2a and 6.2b. These results further suggest that swelling of Dowex 50W x8 resin with ionic strength is not significant enough to affect the selectivity of the resin over the ionic strength range of normal urine samples.

6.3.4 Comparison of Selectivity Constants Obtained Using Chloride and Nitrate Salts

A data treatment similar to that used for Dowex 50W x2 in the previous chapter was applied to the results obtained with Dowex 50W x8. The results of this treatment are given in Table 6.3, which shows that the average selectivity constants for the chloride and nitrate systems are similar, but significantly different for some of the values. Although the relative uncertainties in these values are fairly large($\approx$10%), the same trend is observed here as in Table 5.5 for Dowex 50W x2 resin. The differences in the selectivity constants further suggest that an ion pairing effect may exist.

Table 6.2a Comparison of experimental and predicted K^{mix} values for calcium equilibria in nitrate system with Dowex 50W x8 resin.

Solution number	$K^{mix}_{Ca/Na}$		$K^{mix}_{Ca/K}$	
	Expt.	Pred.	Expt.	Pred.
1	675	705	164	181
2	750	755	194	199
3	783	803	217	227
4	883	824	268	241
5	757	715	178	169
6	725	748	197	203
7	727	737	211	213
8	752	762	264	263
9	620	634	161	159
10	638	651	179	174
11	645	634	242	245
12	654	661	266	268
13	564	559	159	157
14	579	563	184	182
15	613	618	214	216
16	622	629	251	251
17	686	673	221	215
18	726	720	212	217
19	754	749	245	242
20	775	783	274	275
RSEE		3.1%		4.1%
R		0.965		0.973

Table 6.2b Comparison of experimental and predicted K^{mix} values for magnesium equilibria in nitrate system with Dowex 50W x8 resin.

Solution number	$K^{mix}_{Mg/Na}$		$K^{mix}_{Mg/K}$	
	Expt.	Pred.	Expt.	Pred.
1	276	302	67	78
2	318	321	82	85
3	338	340	94	96
4	383	349	116	102
5	310	299	73	70
6	293	310	79	84
7	317	322	92	93
8	332	333	117	115
9	271	272	70	68
10	290	283	81	76
11	285	284	107	110
12	282	298	115	121
13	259	250	73	70
14	249	255	79	82
15	283	274	99	96
16	288	282	116	113
17	305	295	98	94
18	311	314	91	94
19	332	330	108	107
20	347	349	122	122
RSEE		4.2%		5.7%
R		0.924		0.957

Table 6.3 Comparison of averages of selectivity constants calculated using chloride and nitrate salts for calibration of a column containing Dowex 50W x8 resin. The uncertainties in these values are estimated to be of the order of 5 to 10% relative.

K expression	Chloride as anion	Nitrate as anion
$K_{Ca/Mg}$	2.53	2.29
$K_{K/Na}$	1.88	1.81
$K^{mix}_{Ca/Na}$	692	692
$K^{mix}_{Ca/K}$	197	212
$K^{mix}_{Mg/Na}$	273	302
$K^{mix}_{Mg/K}$	78	92

6.3.5 Application of the Method to the Determination of Free Calcium and Magnesium Concentrations in the Presence of Citrate as Complexing Ligand

After the column was calibrated, a coefficient matrix was prepared and used to determine free calcium and magnesium concentrations in test samples. For this purpose a system containing citrate ion as complexing ligand was studied as in the previous chapter to see whether free calcium and magnesium, instead of total concentrations, could be measured. As before, experimental free calcium and magnesium concentrations were compared with calculated values to evaluate the method.

Sample solutions were prepared containing varying amounts of the chloride salts of sodium, potassium, calcium, and magnesium, along with potassium citrate as complexing ligand, in 25-ml volumetric flasks. The pH of the samples was measured with a pH meter for the calculation of the free calcium and magnesium concentrations as before. The composition and pH values of each solution are listed in Table 6.4. Each solution was then pumped through the calibrated column and eluted, after washing, with 2 M HNO_3 into a 10-ml flask. The eluent was diluted to 10 ml with water and introduced into an ARL 34000 ICP-AES instrument for determination of the quantity of metal ions exchanged on the resin phase. Since the stability constants of sodium and potassium with citrate are small, the free sodium and potassium concentrations could be taken as equal to their total concentrations. (Calculations showed more than 99.7% of the sodium and potassium to be in the free form.). Experimental free calcium and magnesium concentrations were calculated as before from a coefficient matrix obtained by calibration of the column with chloride salts, the free sodium and potassium concentrations in each solution, and the metal ion concentrations in the resin phase. Theoretical free calcium and magnesium concentrations were calculated from total ligand and total metal ion concentrations, literature stability constants, and the pH values of the solutions. From the results, shown in Table 6.5, we see that the experimental average values for free calcium and magnesium are reasonably close to the theoretically calculated values.

The above experiment was repeated, using nitrate in place of chloride to prepare the solutions. The experimental method and the data processing procedures were identical to those used with the chloride system. The composition and pH of the solutions are shown in Table 6.6, and the experimental results in Table 6.7. It can be seen that agreement between the experimental and calculated values is quite good.

6.3.6 Investigation of Potential Interference From Zinc(II) and Urea Using the Dowex 50W x8 Column

Several inorganic and organic species normally present in urine were studied to see whether they interfered in the determination of free calcium. Interferences could take several forms. One kind is from ions other than the four major ones that could be present in sufficiently high concentrations or with sufficient affinity for the resin that they compete for resin sites. These could be multicharged cations other than calcium or magnesium, such as zinc, that sorb strongly onto the resin phase, or they could be singly charged ions other than sodium or potassium that are present is appreciable concentrations, such as ammonium ion. Another kind of interference may come from organic substances such as urea, which are present in relatively high concentrations (0.2 to 0.5M) in urine and which could interfere by affecting the properties of the mobile phase in the resin.

Potential interference from ammonium ion has been corrected for by including it in the standards used to calibrate the column. However, possible interference from multiply charged ions such as zinc(II), the polyvalent cation present in highest concentration in urine after calcium and magnesium, or from non-ionic organic species such as urea, needed to be studied. Therefore both zinc(II) and urea were investigated for possible interferences.

Table 6.4 Composition and pH values of solutions prepared from chloride salts for study of the citrate system. All concentrations are given in mM.

Soln. no.	Na	K	Ca	Mg	K3citrate	pH
1	100	100	2.8	4.0	3.0	4.95
2	100	100	4.2	4.0	6.0	4.97
3	200	40	4.2	6.0	3.0	4.82
4	200	40	2.8	4.0	6.0	5.01
5	200	100	2.8	6.0	3.0	4.86
6	200	100	4.2	6.0	6.0	4.88

Table 6.5 Comparison of calculated and experimentally measured concentrations of free calcium and magnesium ion in the presence of citrate as ligand, using a Dowex 50W x8 column. All concentrations given in mM. (Chloride salts used for calibration of column.)

Experimentally determined values			Calculated values	Total concn
(Na)	(K)	Average		
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
1.9	1.9	1.9	2.1	2.8
2.4	2.4	2.4	2.6	4.2
3.1	3.3	3.2	3.5	4.2
1.6	1.6	1.6	1.7	2.8
2.0	2.2	2.1	2.3	2.8
2.7	2.8	2.8	2.9	4.2
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
2.7	2.7	2.7	3.2	4.0
2.5	2.5	2.5	2.7	4.0
4.6	4.8	4.7	5.2	6.0
2.3	2.3	2.3	2.6	4.0
4.5	4.8	4.6	5.1	6.0
4.0	4.2	4.1	4.4	6.0

Table 6.6 Composition and pH values of solutions prepared from nitrate salts for study of the citrate system. All concentrations are given in mM.

Soln. no.	Na	K	Ca	Mg	K_3citrate	pH
1	100	100	3.2	4.0	3.0	4.92
2	100	100	4.8	4.0	6.0	4.92
3	200	40	4.8	6.0	3.0	4.78
4	200	40	3.2	4.0	6.0	4.97
5	200	100	3.2	6.0	3.0	4.83
6	200	100	4.8	6.0	6.0	4.85

Table 6.7 Comparison of calculated and experimentally measured concentrations of free calcium and magnesium ion in the presence of citrate as ligand, using a Dowex 50W x8 column. All concentrations given in mM. (Nitrate salts used for calibration of column.)

Experimentally determined values			Calculated	Total
(Na)	(K)	Average	values	concn
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
2.5	2.6	2.5	2.5	3.2
2.9	3.1	3.0	3.1	4.8
3.7	4.2	4.0	4.0	4.8
1.7	1.9	1.8	2.0	3.2
2.5	2.8	2.6	2.6	3.2
3.1	3.5	3.3	3.4	4.8
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
3.1	3.3	3.2	3.3	4.0
2.5	2.8	2.6	2.8	4.0
4.9	5.6	5.3	5.2	6.0
2.2	2.4	2.3	2.7	4.0
4.7	5.4	5.1	5.2	6.0
4.0	4.6	4.3	4.5	6.0

Sample solutions were prepared by adding varying amounts of $Zn(NO_3)_2$ solution or solid urea, along with fixed amounts of solutions of the chloride salts of sodium, potassium, calcium and magnesium, to 25-ml volumetric flasks. The concentrations of sodium, potassium, calcium and magnesium in each final solution were 0.1 M, 0.05 M, 1.7 mM and 2.0 mM respectively. The solutions were then analyzed for free calcium and magnesium as before. The results are shown in Table 6.8.

From Table 6.8 we see that zinc and urea, when present at concentrations found in normal urines, do not interfere with the measurement of free calcium and magnesium concentrations. In the case of zinc it is likely that even though most or all of the metal is sorbed on the resin, the amount present is sufficiently small that the amounts of the four major ions (Na, K, Ca, Mg) exchanged on the resin phase are not significantly affected, and so values obtained for the exchange constants (K^{mix}) of the system remain unchanged. As for urea, it does not affect ionic equilibria between the solution and resin phases even though present in concentrations as high as 0.5 M. This shows the insensitivity of the ion exchange process to the presence of non-ionic solutes.

6.3.7 Determination of Free Calcium and Magnesium in Urine Samples Using Dowex 50W x8 Resin

In Chapter 4 a urine sample from a normal individual was analyzed using a column containing 25 mg of Amberlyst 15 resin. One disadvantage of that resin was that when urine samples were pumped through it without filtering, particles present in the samples tended to plug the column. Since filtration was slow and tedious, it was of interest to determine whether a column constructed with Dowex 50W x8 resin would require this step. As will be seen, no blocking was found when passing urine samples through a column containing 6.5 mg of resin.

Table 6.8 Effect of Zn^{2+} and urea on the determination of free Ca^{2+} and Mg^{2+} with a column of Dowex 50W x8 resin. All concentrations given in mM.

Soln. no.	Addition	Ca^{2+} (Na)	Ca^{2+} (K)	Ca^{2+} av.
1	None	1.7	1.7	1.7
2	1 ppm Zn^{2+}	1.6	1.6	1.6
3	2 ppm Zn^{2+}	1.9	1.9	1.9
4	10 ppm Zn^{2+}	1.8	1.8	1.8
5	0.1 M urea	1.8	1.7	1.8
6	0.3 M urea	1.6	1.5	1.6
7	0.5 M urea	1.8	1.7	1.7
8	None	1.9	1.8	1.9

Soln. no.		Mg^{2+} (Na)	Mg^{2+} (K)	Mg^{2+} av.
1	None.	1.9	1.9	1.9
2	1 ppm Zn^{2+}	1.8	1.9	1.8
3	2 ppm Zn^{2+}	2.2	2.2	2.2
4	10 ppm Zn^{2+}	2.0	2.0	2.0
5	0.1 M urea	2.1	2.0	2.0
6	0.3 M urea	1.9	1.8	1.8
7	0.5 M urea	2.0	1.9	2.0
8	None	2.2	2.1	2.1

Fresh urine samples were collected from four healthy volunteers and analyzed within one hour. The pH of each sample was measured with a pH meter. A portion of each sample was step diluted for determination of total S, P, Ca, Mg, Na and K by ICP-AES as follows: (1) a 2-ml aliquot of the urine was delivered to a 10-ml volumetric flask, 1 ml of 2 M HNO_3 added, and the solution diluted to 10 ml. This solution, labeled A, was used for determination of total S and P. (These analyses were performed in order to estimate the concentrations of sulphate and phosphate, which were used to obtain the free Na and K concentrations by equilibrium calculations from the total Na and K concentrations, the sulphate and phosphate concentrations and the pH of the solution.) (2) 2 ml of solution A was delivered to a 10-ml volumetric flask, 0.8 ml of 2 M HNO_3 added, and the solution diluted to 10 ml. This solution, labeled B, was used for the determination of total calcium and magnesium. (3) 2 ml of solution B was delivered to a 10-ml volumetric flask, 0.8 ml of 2 M HNO_3 added, and the solution diluted to 10 ml. This solution, labeled C, was used for the determination of total sodium and potassium. The remaining urine sample was pumped through the column of ion exchange resin to equilibrium and the sorbed ions eluted and measured as described before.

From the results, shown in Table 6.9, we can see that the four urine samples vary considerably in pH, and in the total concentrations of sodium, potassium, calcium, and magnesium, as well as in the percentages of calcium and magnesium present in free form. The fractions of free calcium and magnesium found ranged from 40% to 66% in these four samples. This is within the estimated range reported for free calcium and magnesium in urine, which states that approximately 50% of the total Ca and Mg can be expected to be present in the free form [22-27]. The analyses described here require less than 30 minutes per sample, and have a relative error estimated to be of the order of 10% or less.

Table 6.9 Determination of free calcium and magnesium in normal urine samples

Sample number and pH	Total conc. from ICP	Free Ion Concentration from Ion-Exchange		
		calc'd fm Na conc.(ppm)	calc'd fm K conc.(ppm)	Average
1	Ca 316 ppm	116, 129	125,133	126 ppm (40%)[a]
	Mg 105 ppm	42, 47	46, 48	46 ppm (44%)[a]
pH 5.69	Na 0.251 M			0.239 M[b]
	K 0.062 M			0.059 M[b]
	S 0.033 M			
	P 0.023 M			
2	Ca 218 ppm	123, 134	157,159	143 ppm (66%)[a]
	Mg 45 ppm	25, 27	32, 32	29 ppm (64%)[a]
pH 6.29	Na 0.250 M			0.244 M[b]
	K 0.023 M			0.023 M[b]
	S 0.013 M			
	P 0.009 M			
3	Ca 154 ppm	91, 100	88, 96	94 ppm (61%)[a]
	Mg 68 ppm	43, 47	42, 45	44 ppm (65%)[a]
pH 5.28	Na 0.219 M			0.209 M[b]
	K 0.051 M			0.049 M[b]
	S 0.025 M			
	P 0.025 M			
4	Ca 134 ppm	62, 66	61, 65	63 ppm (47%)[a]
	Mg 126 ppm	66, 68	65, 68	67 ppm (53%)[a]
pH 5.74	Na 0.207 M			0.199 M[b]
	K 0.069 M			0.066 M[b]
	S 0.024 M			
	P 0.019 M			

[a] Values in parentheses are percentages of the total concentration present in the free ion form.

[b] Free ion concentrations calculated from their total concentrations, corrected for complexation by sulphate and phosphate at the urine pH.

6.3.8 Application of the Method to the Determination of Free Calcium and Magnesium Concentrations in a Freeze-Dried Standard Reference Urine Sample

From the previous section, we see that the method is applicable to typical urine samples. There is no simple way, however, to determine the accuracy of the results because there is no reliable method with which to compare it. It was thought worthwhile, therefore, to analyze a generally available material that could provide a basis for comparison with other methods in the future. The U.S. National Institute of Standards and Technology provides such a material in the form of a standard reference urine sample, SRM 2670 (Toxic Metals in Freeze-Dried Urine). An additional reason for analyzing this standard, which is certified for total sodium, calcium, and magnesium (a non-certified value for total potassium is given for information only), is that it was analyzed for free calcium and magnesium by a previous worker in this laboratory, A. Hewavitharana, using an earlier version of the ion-exchange equilibrium method [16]. She also analyzed this SRM for free calcium colorimetrically using tetramethylmurexide [16]. Therefore it was of interest to compare results obtained by these different methods.

SRM 2670 is provided in the form of four bottles of freeze-dried urine, two containing low and two elevated levels of toxic metals. The low level material is normal human urine that was prepared from pooled samples. The elevated level is a pooled normal human urine that was spiked with selected metals. The certified total concentration values of the constituent elements in SRM 2670 are shown in Table 6.10 No information on free metal concentrations was provided by NIST. Zinc, one of the trace elements present in higher concentrations in normal urine, could not be certified in SRM 2670 due to contamination from the stopper used in the packaging. In reconstituted samples, the zinc concentration varied from 0.5 to 1.5 ppm.

Table 6.10 Certified values of constituent elements in NIST SRM 2670 (The values provided in parentheses are not certified but are given by NIST for information only)

Element	Conc. units	Normal level		Elevated level	
Al	ppm	(0.18)		(0.18)	
As	ppm	(0.06)		0.48	±0.10
Be	ppm	(<0.0005)		(0.033)	
Cd	ppm	(0.00040)		0.088	±0.003
Ca	ppm	105	±5	105	±5
Cl	ppm	(4400)		(4400)	
Cr	ppm	(0.013)		0.085	±0.006
Cu	ppm	0.13	±0.02	0.37	±0.03
Au	ppm	(0.000008)		(0.24)	
Pb	ppm	(0.01)		0.109	±0.004
Mg	ppm	63	±3	63	±3
Mn	ppm	(0.03)		(0.33)	
Hg	ppm	(0.002)		0.105	±0.008
Ni	ppm	(0.07)		(0.30)	
Pt	ppm	(0.000008)		(0.12)	
K	ppm	(1500)		(1500)	
Se	ppm	0.030	±0.008	0.46	±0.03
Na	ppm	2620	±140	2620	±140

The urine standards were reconstituted by the addition of 20.0 ml of pure water to each bottle, then analyzed in the same way as described in the previous section for normal urine samples. The results are shown in Tables 6.11a and 6.11b, together with the results of A. Hewavitharana in this laboratory for comparison.

From Tables 6.11a and 6.11.b, we see that the percentage of free Ca and Mg found here are quite different from the results obtained by the ion exchange/AA method used previously [16], while the free Ca results from this experiment are reasonably close to those found using the TMMA colorimetric method [16].

The following possibilities were proposed in reference [16] to explain the discrepancy between the free calcium levels found by the ion exchange/AA and TMMA colorimetric methods: (1) Interference from other metal ions in SRM 2670. It was pointed out that the differences in concentrations of heavy metals in the normal and elevated samples is large, while the free calcium results for these two samples vary little. The two methods indeed show opposite deviations between the normal and the elevated samples, although the differences are not large. It was concluded that interference from other metals, even if present, was not likely to be the major cause of the difference in free calcium values seen with the two methods. (2) Improper matching of electrolytes in samples and standards. It was pointed out that the potassium concentration reported for SRM 2670 is not a certified value, and also, if any significant concentration of ammonium ion was present in the sample, the effect would not be accounted for. A study of the effect of potassium concentration on the two methods showed that the free calcium concentration decreases for both methods as the potassium concentration increases. The conclusion is that any difference in free calcium concentrations measured by the two methods that can be ascribed to mismatched ionic strength is not likely to be very large. (3) Interference from organic substances. The ion exchange studies done previously have shown that measurements of free calcium are not affected by the presence of urea at concentrations

Table 6.11a Results of analysis of normal level (non-spiked) sample of NIST urine standard SRM 2670 for free calcium and magnesium[a]

Total conc. found from ICP	Free ion conc. (based on Na^+ calc.) (ppm)	Free ion conc. (based on K^+ calc.) (ppm)	Ave free ion found
Normal level (non-spiked) sample, pH 4.60, this work			
Ca 108 ppm	70, 73, 67, 71	71, 71, 68, 69	70 ppm (65%)
Mg 61 ppm	42, 44, 41, 42	42, 43, 41, 41	42 ppm (69%)
Na 0.106 M			0.102 M[b]
K 0.037 M			0.036 M[b]
S 0.016 M			
P 0.017 M			
Ref. [16]	Ion exchange/AA		Ca 51ppm (49%) Mg 31ppm (49%)
	TMMA colorimetric		Ca 72ppm (69%)

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[a] Each sample was passed twice through the column to give duplicate solutions for analysis. Each eluent was analyzed in duplicate by ICP-AES.

[b] Free ion concentrations calculated from their total concentrations, corrected for complexation by sulphate and phosphate at the urine pH.

Table 6.11b Results of analysis of elevated level (spiked) sample of NIST urine standard SRM 2670 for free calcium and magnesium[a]

	Total conc. found from ICP	Free ion conc. (based on Na$^+$ calc.) (ppm)	Free ion conc. (based on K$^+$ calc.) (ppm)	Ave free ion found
Elevated level (spiked) sample, pH 4.43, this work				
Ca	107 ppm	74, 77, 69, 76	76, 77, 68, 75	74 ppm (69%)
Mg	59 ppm	43, 45, 41, 44	44, 44, 40, 44	43 ppm (73%)
Na	0.106 M			0.102 M[b]
K	0.037 M			0.036 M[b]
S	0.015 M			
P	0.016 M			
Ref. [16]		Ion exchange/AA		Ca 46ppm (44%)
				Mg 28ppm (44%)
		TMMA colorimetric		Ca 75ppm (71%)

[a] Each sample was passed twice through the column to give duplicate solutions for analysis.

Each eluent was analyzed in duplicate by ICP-AES.

[b] Free ion concentrations calculated from their total concentrations, corrected for complexation by sulphate and phosphate at the urine pH.

similar to that in urine[142]. Studies of possible interference from urea with the spectrophotometric TMMA method have not been reported. (4) Interference from background absorbance of urine with the spetrophotometric method. Usually urine is used as a reference for the colorimetric method, but when absorption by urine is high the measurement is less accurate. (5) Comparison of measured with expected fractions of free calcium and magnesium. According to the literature, about 50% of the calcium in normal urine can be expected to be found in the free form. The ion exchange/AA method yielded free calcium and magnesium levels close to 50%, while the TMMA method gave values that were about 20% higher.

The above discussion in general supports the results obtained by the ion exchange method here, although no firm conclusions can be drawn. Further, inference as to whether free calcium concentrations measured using the ion exchange method are more reliable than those obtained using the spectrophotometric method is not possible without further study.

The ion exchange/ICP method proposed here, however, produced free calcium levels that were reasonably close to those of the TMMA colorimetric results, and quite different from the ion exchange/AA method. So which method is more accurate and reliable? The following points in general support the ion exchange/ICP method.

A. Probability of interference from ammonium ion in the ion exchange/AA method.

Ammonia usually is present in urine in the range of 0.02 to 0.06 M; over the normal urine pH range of 4 to 7, the major form is ammonium ion. In the ion exchange/ICP method ammonium ion does not interfere because it is included in the calibration standards at concentrations from 0.02 to 0.1 M. In this way possible interference with the selectivity constants of the metal ions has been accounted for. On the other hand, interference from ammonium ion on the ion exchange/AA method was not investigated, but can be expected to occur. The key equations applied in the ion exchange/AA method were [16]:

$$[R_2Ca]^2 = a[Ca] + b[Ca][Mg]$$

and

$$[R_2Mg]^2 = p[Ca] + q[Mg] + r[Ca]^2 + s[Mg]^2 + t[Ca][Mg]$$

In these expressions $[R_2Ca]$ and $[R_2Mg]$ represent the amounts of calcium and magnesium in the resin phase, [Ca] and [Mg] represent the free calcium and magnesium concentrations in the solution phase, and a, b, p, q, r, s and t are coefficients obtained for each of the free metal ion concentration terms. When ammonium ions are present, they will exchange with ions on the resin phase, and the amount of calcium and magnesium in the resin phase will decrease. From reference[16], we see that a is much larger than b ($a = 0.02792$, $b = -0.00016$) and q is larger than p, r, s and t ($q = 0.1481$, $p = -0.0384$, $r = 0.00076$, $s =0.0021$, $t = -0.0026$), so values of [Ca] and [Mg] change nearly as the square of $[R_2Ca]$ and $[R_2Mg]$.

To estimate the effect of ammonium ion, we know from calibration of the Dowex 50W x8 column in this chapter that the column capacity is about 23.8 microequivalents. When the equilibration solution is made 0.5 M in ammonium ion, the total amount of the four major ions (Na, K, Ca and Mg) found in the resin phase at equilibrium decreases to 19.5 microequivalents. Since $[R_2Ca]$ and $[R_2Mg]$ decrease to about 82% if we assume that all four major metals in the resin phase are reduced in the same proportion, then [Ca] and [Mg] decreased to about 67%. Upon applying this correction factor to the ion exchange/AA result, we get free calcium levels of 73% and 66% for normal and elevated samples, respectively. These results bring this method into closer agreement with the other two methods. Although this is only a rough estimation and makes a number of assumptions, the probability of ammonium interference with the ion exchange/AA method is apparent.

B. The trend of free calcium and magnesium percentages between normal and elevated metal levels in SRM 2670

From table 6.11 we see that for both the ion exchange/ICP and TMMA colorimetric methods, the free calcium and magnesium percentages are higher for the samples with elevated metal levels, while the opposite trend is seen for the ion exchange/AA method.

In the ion exchange/AA method, since heavy metal ions tend to be sorbed more strongly on the resin for a given charge, elevated levels of these ions will result in less calcium and magnesium being taken up by the column. This means that measured free calcium and magnesium levels by this method will be lower in the samples containing elevated metals than they should be. In the ion exchange/ICP method, on the other hand, the free calcium and magnesium concentrations are calculated relative to free sodium and potassium concentrations, so that even though part of the column capacity may be taken up by heavy metal ions, and the amounts of the four major ions (Ca, Mg, K, Na) on the resin phase are smaller, the calculated calcium and magnesium concentrations would still be correct as long as the uptake of other ions is not so high that it alters the selectivity constants between the major ions. For this reason the ion exchange/ICP method is better able to tolerate the presence of other cations. As was seen from the zinc interference study, levels of zinc as high as 10 ppm do not interfere.

It was pointed out previously that the vials of SRM 2670 containing elevated levels of toxic metals consisted of normal human urine spiked with selected metals. The form in which the metals were added was not specified. If simple salts of the selected metals were used, as seems likely, then these metal ions would compete with calcium and magnesium for complexing ligands present in the urine sample. This would result in more free calcium and magnesium in the spiked urine samples. This is in agreement with the trend found by both ion exchange/ICP and TMMA colorimetry, but not by ion exchange/AA.

From the above discussion, it can be concluded that the ion exchange/ICP method is likely to give more accurate results than the ion exchange/AA method. Definitive proof would, however, require further studies.

6.4 *Conclusions*

In this chapter, a column of Dowex 50W x8 resin was used for the determination of free calcium and magnesium concentrations by the previously developed ion exchange/ICP method. Results with this resin were more satisfactory than with the resins studied earlier. Unlike Amberlyst 15, no blocking or plugging of the column was observed when urine samples were passed through it. And unlike Dowex 50W x2, calculations were simpler and more precise because swelling effects did not have to be taken into account.

Four urine samples from normal individuals were analyzed for free calcium and magnesium. The percentages found ranged from 40 to 65%. A standard freeze dried urine (NIST SRM 2670) containing normal and elevated metal levels was also analyzed. The levels of free calcium found were closer to previous results obtained by a colorimetric method using tetramethylmurexide than to one using ion exchange/AA.

Advantages of the proposed ion exchange/ICP method over the ion exchange/AA method include simplicity and reduced interferences. One set of standards is sufficient for calibration of the column, and a range of urine samples having widely varying compositions can be analyzed without need of matching standards to samples. About 30 minutes are required to analyze one urine sample. This time per sample could likely be reduced if multiple samples were being analyzed. Work with a reference standard containing elevated amounts of toxic metals indicates that the method can tolerate reasonable levels of other cations without interference. The ion exchange/ICP method therefore appears to be promising for the clinical determination of free calcium and magnesium concentrations in urine samples.

Chapter 7 Investigation of On-Line ICP for Determination of Metal Ions Eluted from Resin Phase in Ion-Exchange Equilibration Procedure

7.1 *Introduction*

In the previous chapters, three ion-exchange resin types were investigated for the determination of free calcium and magnesium concentrations in urine samples by the ion exchange/ICP method. Dowex 50W x8 was found to be the most useful resin; using it, urine samples and a standard reference material were successfully analyzed. In all these studies the metal ions in the resin phase were determined by first eluting them from the column into volumetric flasks, and then taking them to an ICP-AES instrument for measurement. In this chapter, experiments are described in which the column was connected directly to the nebulizer of the ICP-AES instrument to investigate the feasibility of on-line determination. A similar on-line approach has been used before in the ion exchange/AA method [16].

7.2 *Column Constraction and Chemicals*

A new column, prepared in the same way as the previous ones but containing 2.1 mg of Dowex 50W x8 resin was used for these experiments. All chemicals and solutions were the same as those used in previous chapters.

7.3 *Experimental Apparatus and Procedure*

The experimental setup was the same as described previously except for the method of eluent collection. Instead of collecting the eluent from the column in a volumetric flask, the tube leading from the column was connected directly to the sampling tube of the ARL ICP-AES instrument through a rotary sample injection valve. The injection valve was used to inject standards for calibrating the quantity of metal ions eluted from the resin phase as well as to inject urine samples for simultaneous determination of total Na, K, Ca, Mg, S and P. A diagram of the system is shown in Fig. 7.1.

7.3.1 Experimental Procedure for Determination of the Amount of Metal Ions Eluted From the Resin Phase

1. Set sample injection valve D to load position (solid line in Figure 7.1). Using the syringe, fill the sample loop with standard solution and start the ICP-AES integration either immediately before or after the injection. Continue data acquistion for 100 seconds. Normally two standards were used, one containing higher, and the other lower, concentrations of each of the four metal ions of interest (Na, K, Ca and Mg). Standards and samples were run in alternation so that each sample was bracketed by a high and a low standard.

2. Pump sample through the column until equilibration is achieved between the resin and solution phases (Three minutes was found sufficient. See Section 7.5). Pass air through the column to remove residual solution (about a minute is sufficient), then wash the column with water for about a minute. Elute the metal ions from the resin phase with 2 M HNO_3 for one minute by turning valve C to eluent position; start the ICP-AES integration of peak areas 30 seconds after opening the valve for elution. Continue to collect data until the signal has returned to the base line (100 seconds was used).

7.3.2 Experimental Procedure for Determination of Total Concentrations of Species of Interest in Urine Samples

With the sample injection valve, alternately inject standards and unknown urine samples and collect data for the determination of total Na, K, Ca, Mg, P and S, bypassing

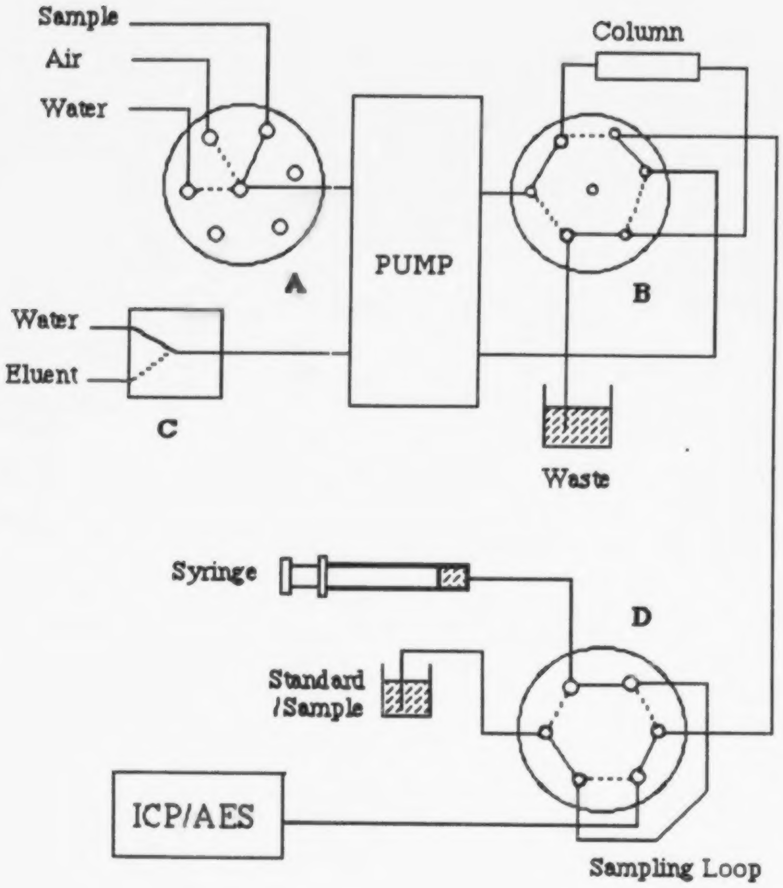

Sample
Air
Water
Column
PUMP
A
B
Water
Eluent
C
Waste
Syringe
D
Standard /Sample
ICP/AES
Sampling Loop

Figure 7.1 Diagram of flow system from ion-exchange column to ICP-AES instrument for on-line analysis of column eluent.

the resin column. The procedure used in this study was to record integrated signals from the ARL ICP-AES instrument for each element simultaneously for 100 seconds. These data were then used to calculate the total concentrations of the various species present in the urine samples, along with the free magnesium and calcium concentrations, as before.

7.4 *Determination of Integration Time Needed for ICP-AES Measurements*

With the flow system connected directly to the ICP-AES instrument, metal ions eluted with strong acid from the column will first pass through the connecting tubes, then through the nebulizer and transportation tube in the instrument before reaching the plasma. Some time is therefore required for all the metal ions in the resin phase to be eluted out of the column and carried through the nebulizer to the torch. Unfortunately, the ARL-34000 does not have provision to provide a continuous response signal with respect to time. Therefore a series of 2-second integrations were collected to monitor the profile. An approximately 5 or 6 second interval is necessary between integrations. Since the nebulizer has a volume of about 30 ml, some time is required for the signal to reach a stable new level when the concentration of analyte is changed. This produces considerable band broadening of the signal when a sample is introduced with the injection valve or when eluted from the column. Profiles of the four elements of interest in the column eluent were determined both from the injection valve and the column with a single standard solution containing 0.1 M NaCl, 0.05 M KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. The results are shown in Figures 7.2a to 7.2d. An integration time of 100 seconds was used for subsequent determinations to make sure that all the signal from the results of the profiles was integrated by the detection system .

The long integration time required by this procedure is a drawback. For the reasons discussed above the integration time could not be reduced significantly. The effect of this

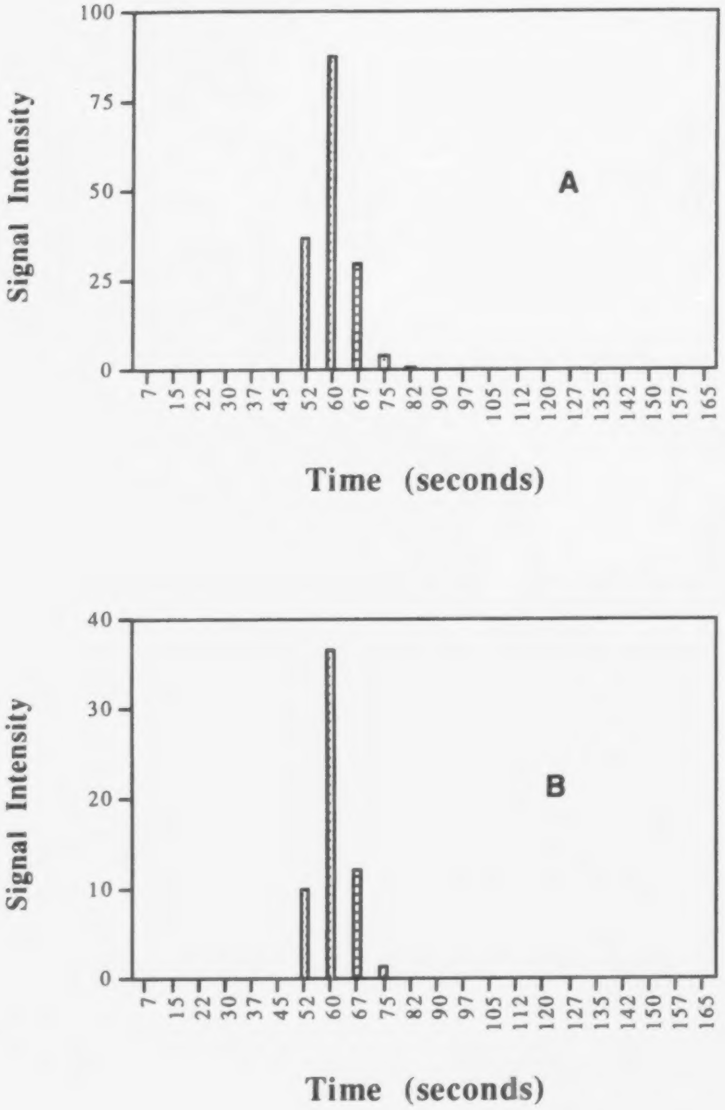
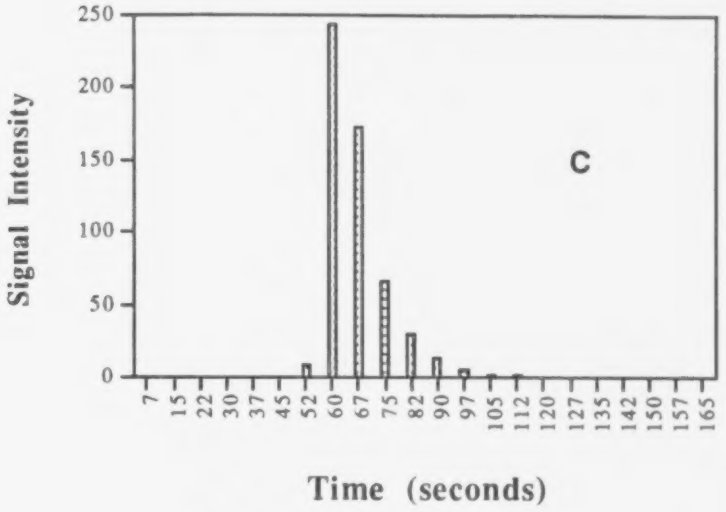


Figure 7.2a Profile of solutions of column eluent pumped directly into ICP nebulizer (plot A, sodium; plot B, potassium). Starting time is at switching of valve to initiate elution of column with 2 M nitric acid. In later studies, signals were integrated for 100 seconds, from the 30th to the 130th second, to ensure all of the signal was collected.



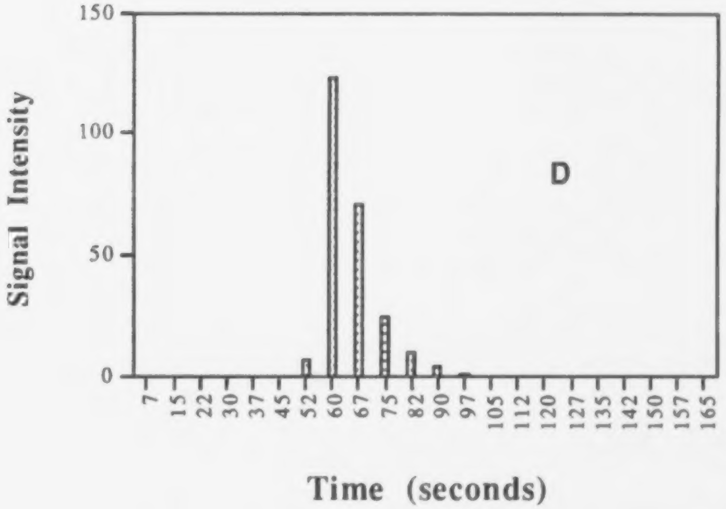
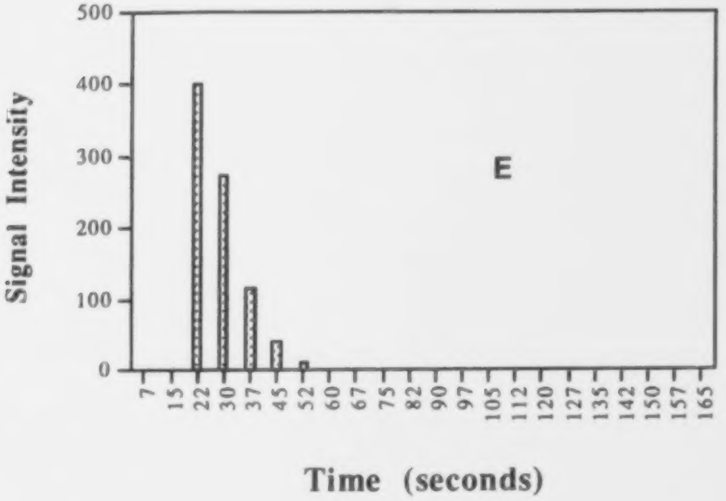


Figure 7.2b Profile of solutions of column eluent pumped directly into ICP nebulizer (plot C, calcium; plot D, magnesium). Starting time is at switching of valve to initiate elution of column with 2M nitric acid. In later studies, signals were integrated for 100 seconds, from the 30th to the 130th second, to ensure all of the signal was collected.



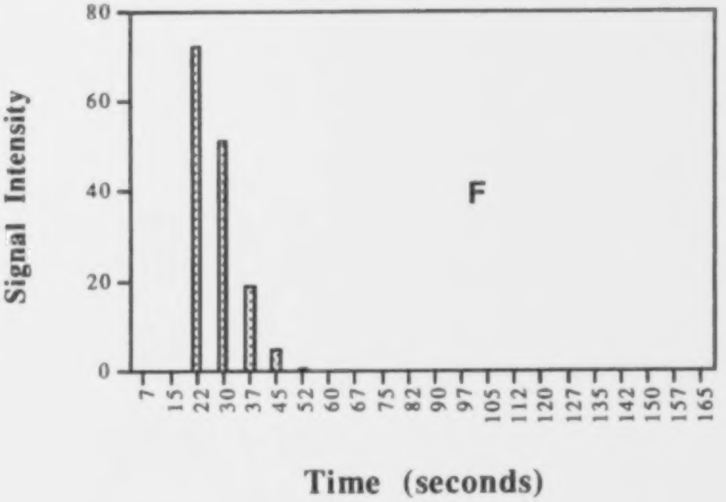
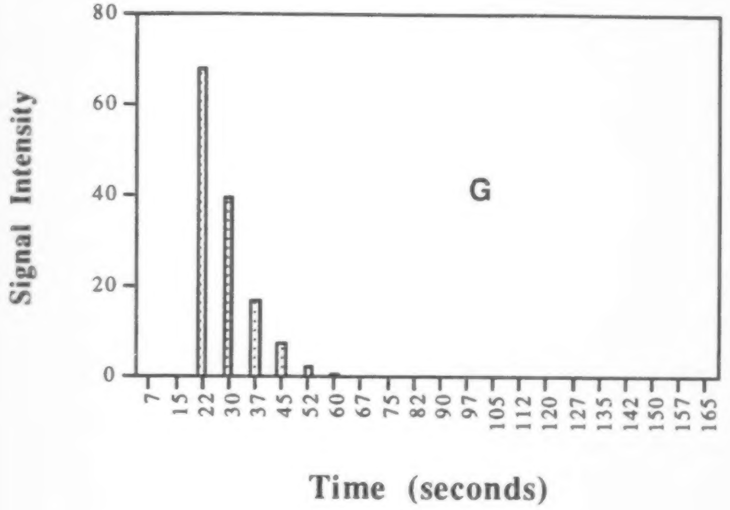


Figure 7.2c Profile of samples collected using sample injection valve (plot E, sodium; plot F, potassium). Starting time is at switching of valve to initiate injection of standard solution. For later studies the signals were integrated for 100 seconds, starting at the time the valve was switched, to ensure all the signal was collected.



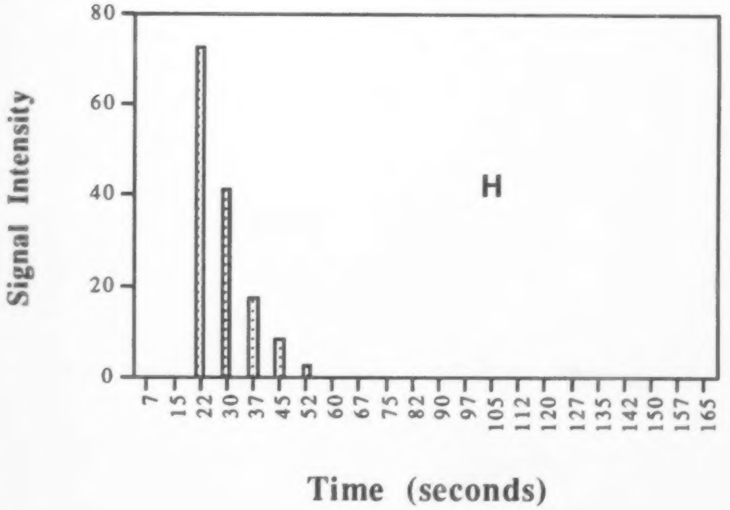


Figure 7.2d Profile of samples collected using sample injection valve (plot G, calcium; plot H, magnesium). Starting time is at switching of valve to initiate injection of standard solution. For later studies the signals were integrated for 100 seconds, starting at the time the valve was switched, to ensure all the signal was collected.

is that the measurement takes longer, and is subject to decreased accuracy if the instrument drifts with time.

7.5 *Equilibration Time Study*

From the results of experiments described in the previous chapter we know that for a column with 6.4 mg Dowex 50W x8 resin, 3 minutes was sufficient for equilibration of the column, and 1 minute was enough for elution. Here, since the column contains only 2.1 mg of Dowex 50W x8 resin, the 3-minute equilibration and 1-minute elution times should be ample. To confirm this, a standard having lower concentrations of the chloride salts of sodium, potassium, calcium, and magnesium was prepared and evaluated. The concentrations of the four ions in the standard were: Na, 60 mM; K, 20 mM; Ca, 0.8 mM; and Mg, 0.8 mM. The elution time was fixed at 1 minute, and the equilibration time varied from 1 to 10 minutes. The elution profile, shown in Figure 7.3, confirms that 3 minutes is sufficient for equilibration. Further studies were not considered to be necessary, especially since the column contained considerably less resin than the column used in the last chapter. In all studies in this chapter, therefore, 3 minutes was used for column equilibration, and 1 minute for column elution.

7.6 *Calibration of Column*

The concentration of metal ions in the resin phase can be defined arbitrarily so long as one column is used, as discussed in previous chapters. Here a convenient unit for use is millimoles of each element in the volume of the sampling loop. The integrated signal from the sample eluted from the resin column was compared with the integrated signal from a standard solution injected with the sample injection valve for each of the four metal ions in the resin phase. This convention will be used throughout the remainder of this chapter.

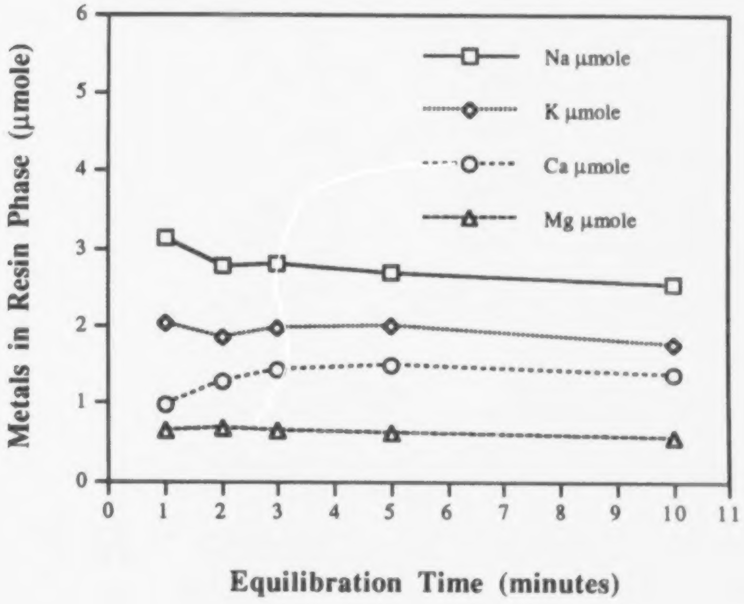


Figure 7.3 Results of equilibration time study for column containing 2.1 mg of Dowex 50W x8 resin connected directly to an ARL 34000 ICP-AES.

A set of standards was prepared from the chloride salts of the metal ions with the concentrations listed in Table 7.1. This set was treated in the usual way, using the established method, and the equilibrium concentrations of the metal ions in the resin phase were measured. Mixed selectivity constants were obtained for each standard and the coefficients calculated by multiple regression with the BASIC program described earlier. The predicted selectivity constants, obtained using the metal ion concentrations found in the resin phase and the calculated coefficients, were compared with the experimental values. Tables 7.2 and 7.3 show the results. From these values, we see that the fits are poorer than those from the last chapter, but remain useful for estimation of free calcium and magnesium levels. The main reason for the poor fit may be due to the long integration times required. Over time instrument drift occurs, leading to degradation of the precision of the results..

7.7 *Application to Determination of free Calcium and Magnesium in Presence of Citrate*

A solution containing citrate was studied as before to assess the applicability of the direct method to the determination of free calcium and magnesium. Sample solutions containing varying amounts of the chloride salts of sodium, potassium, calcium, and magnesium, along with potassium citrate as complexing ligand, were placed in 25-ml volumetric flasks. The pH of each solution was measured for use in equilibrium calculations of the levels of free calcium and magnesium. The compositions and pH values of these solutions are listed in Table 7.4. Each solution was then passed through the calibrated column and eluted with 2 M HNO_3 directly into the ARL model 34000 ICP-AES instrument for the determination of the amount of metal ions exchanged on the resin phase. The stability constants of sodium and potassium with citrate at this pH are small enough that their free ion concentrations could be considered to be equal to their total concentrations

Table 7.1 Composition of standards prepared for calibration of Dowex 50W x8 resin column used in this chapter. All concentrations are given in mM.

Std.	NaCl	KCl	$CaCl_2$	$MgCl_2$	NH_4Cl
1	60	20	0.8	0.8	0
2	60	40	1.6	1.6	0
3	60	80	3.2	4.0	0
4	60	120	4.8	6.0	0
5	100	20	1.6	4.0	0
6	100	40	0.8	6.0	0
7	100	80	4.8	0.8	0
8	100	120	3.2	1.6	0
9	200	20	3.2	6.0	0
10	200	40	4.8	4.0	0
11	200	80	0.8	1.6	0
12	200	120	1.6	0.8	0
13	280	20	4.8	1.6	0
14	280	40	3.2	0.8	0
15	280	80	1.6	6.0	0
16	280	120	0.8	4.0	0
17	200	80	3.2	4.0	0
18	100	40	1.6	1.6	20
19	100	40	1.6	1.6	50
20	100	40	1.6	1.6	100

Table 7.2 Comparison of experimental and calculated K^{mix} values for calcium ion equilibria

Soln.	$K^{mix}_{Ca/Na}$ Values		$K^{mix}_{Ca/K}$ Values	
	Expt.	Calc'd.	Expt.	Calc'd.
1	192	216	43	48
2	225	235	52	54
3	270	242	66	56
4	269	246	69	60
5	233	201	49	43
6	140	190	36	51
7	222	249	59	62
8	222	243	66	76
9	186	186	47	43
10	192	213	46	57
11	167	180	58	59
12	167	198	60	68
13	152	182	41	47
14	217	184	57	50
15	191	172	61	51
16	213	174	65	57
17	238	199	64	53
18	211	214	43	54
19	241	228	69	63
20	292	253	100	85
Correl. coeff.		0.702		0.778
Rel. std. err. of estimate,%		14		16

Table 7.3 Comparison of experimental and calculated K^{mix} values for magnesium ion equilibria.

Soln.	$K^{mix}_{Mg/Na}$ Values		$K^{mix}_{Mg/K}$ Values	
	Expt.	Calc'd.	Expt.	Calc'd.
1	84	93	19	21
2	96	103	22	24
3	110	107	27	25
4	112	109	29	27
5	96	83	20	18
6	61	76	16	20
7	127	113	33	28
8	108	107	32	33
9	76	76	19	18
10	84	90	20	24
11	69	74	24	24
12	64	84	23	29
13	66	76	18	19
14	83	77	22	21
15	83	69	26	20
16	90	71	27	23
17	92	85	25	22
18	85	92	17	23
19	99	98	28	27
20	119	107	41	36
Correl. coefficient		0.820		0.817
Rel. std. error of estimate, %		12		15

Table 7.4. Composition and pH of test system containing citrate. All values are given in mM.

Soln.	Na	K	Ca	Mg	K_3citrate	pH
1	100	100	3.2	4.0	3.0	4.98
2	100	100	4.8	4.0	6.0	5.02
3	200	40	4.8	6.0	3.0	4.90
4	200	40	3.2	4.0	6.0	5.09
5	200	100	3.2	6.0	3.0	4.97
6	200	100	4.8	6.0	6.0	4.98

(theoretical calculations showed more than 99.7% of sodium and potassium to be present in the free form).

Experimental free calcium and magnesium concentrations were calculated from the coefficient matrix obtained through calibration of the column, the sodium and potassium concentrations in each solution, and the metal ion concentrations in the resin phase. The theoretical free calcium and magnesium concentrations were calculated from the total ligand and total metal ion concentrations, the appropriate stability constants, and the pH values of the solutions. The results are shown in Table 7.5.

From the table we see that the average experimental values obtained for free calcium and magnesium are sufficiently close to the calculated values to be considered satisfactory.

7.8 *Analysis of Urine Samples*

Urine samples were collected from two healthy volunteers. The analyses were performed within one hour of collection to minimize changes in concentration from precipitation, air oxidation, etc. The pH of each sample was measured, then the samples analyzed by the procedure described in the previous chapter, but with the column eluent pumped directly into the nebulizer of the ICP-AES. From the results, shown in Table 7.6, it can be seen that the free calcium and magnesium fractions in these two urines range from 54 to 62%.

As mentioned, the long integration time required by this method degrades the precision and accuracy of the analytical results unless the ICP instrument is very stable. In order to reduce band broadening and consequent integration time of the system, one would need to modify the nebulizer and arrangement for sample introduction. A microconcentric nebulizer designed for FIA or HPLC connection with ICP-AES would be one option [144], but the detection limits obtainable with this system are usually higher than the

Table 7.5. Results for determination of free calcium and magnesium in presence of citrate, using direct elution from a Dowex 50W x8 resin column into an ARL 34000 ICP-AES instrument. All concentrations given in mM.

Experimentally determined values			Calculated values	Total concn
(Na)	(K)	Average		
$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	$[Ca^{2+}]$	Ca
2.6	2.8	2.7	2.5	3.2
3.0	3.2	3.1	3.0	4.8
4.4	4.5	4.5	4.0	4.8
1.8	2.0	1.9	1.9	3.2
3.1	3.0	3.0	2.6	3.2
3.5	3.6	3.5	3.3	4.8
$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	$[Mg^{2+}]$	Mg
3.1	3.3	3.2	3.2	4.0
2.4	2.7	2.5	2.7	4.0
5.5	5.6	5.5	5.2	6.0
2.4	2.6	2.5	2.6	4.0
5.5	5.4	5.5	5.0	6.0
4.4	4.4	4.4	4.4	6.0

Table 7.6 Results of analyses of urine samples using a 2.1 mg column of Dowex 50W x8 resin and direct elution of column eluent into an ARL 34000 ICP-AES.

Sample Number and pH	Total Conc. from ICP	Free Ion Concentration from Ion-Exchange		
		calc'd fm Na conc.	calc'd fm K conc.	Average (% of total)
1	Ca 5.2 mM	2.7 mM	2.8 mM	2.8 mM (54%)
pH	Mg 6.1 mM	3.4 mM	3.4 mM	3.4 mM (56%)
6.70	Na 0.222 M			0.217 M[a]
	K 0.071 M			0.069 M[a]
	S 0.018 M			
	P 0.006 M			
2	Ca 9.1 mM	5.3 mM	4.8 mM	5.1 mM (56%)
pH	Mg 7.7 mM	5.0 mM	4.5 mM	4.8 mM (62%)
6.29	Na 0.239 M			0.220 M[a]
	K 0.047 M			0.044 M[a]
	S 0.041 M			
	P 0.048 M			

[a] Free ion concentrations calculated from their total concentrations, corrected for complexation by sulphate and phosphate at the urine pH.

conventional design; also, it is very expensive. Further experiments to reduce band broadening were not performed in this study.

7.9 *Conclusions*

In this chapter, an on-line method for measuring the metal ion concentrations in the resin phase is described. The step of eluent collection with flasks is eliminated. The method has been successfully applied to citrate systems as well as urine samples.

The time required for column equilibration and measurement of the metal ions in the resin phase was similar to that for the indirect method described in the last chapter. A disadvantage of the direct procedure is that the ICP-AES plasma has to be kept operating while the column is being equilibrated with sample solution. This means that considerable quantities of argon gas are consumed between data acquisitions. This situation would be worse with larger columns that required longer equilibration times.

Because a sample injection valve was used for the determination of total Na, K, Ca, Mg, S and P, the viscosity of the sample will normally not affect the accuracy of these measurements. This is because the signals could be collected over a wider integration interval, making step dilution unnecessary. Compared with the indirect method used in the previous chapters, the direct method simplified measurements of the total concentrations of the elements.

It can be concluded that pumping of eluent from a column of Dowex 50W x8 resin directly into the nebulizer of an ARL 34000 ICP-AES is feasible, but the volume of the transport tubing and of the nebulizer itself make the system too slow and expensive for routine use. It may be possible to devise a more direct sample introduction system with reduced dead volume to overcome these disadvantages, but this problem was not pursued further here.

Chapter 8

Conclusions and Suggestions for Future Work

8.1 *Conclusions*

This thesis has explored two methods for free metal ion determinations. The first is a spectrophotometric method, called the indicator increment method (IIM), in which absorbances were measured after multiple addition of different amounts of indicator solution to the same sample solution. Through multiequilibration calculations, the free metal ion concentration in the original solution, as well as the total metal ion and total ligand concentrations, could be estimated. This method could be applied to free metal ion measurements in simple systems where only one type of ligand is present in the sample, and where the type of metal-ligand complex as well as its conditional stability constants are known in advance.

The indicator calmagite was used for measurement of free magnesium by this approach. It was found that other metal ions such as calcium interfere. Also, it was necessary for measurements to be taken within certain pH and ionic strength values. Clearly this method is not suitable for the measurement of free magnesium concentration in complex systems such as urine, where calcium exists at millimolar levels and where pH as well as ionic strength varies considerably from sample to sample. For well defined solutions under controlled conditions, however, the method could be useful, especially if an indicator more selective in its metal coordination, and usable over a wider range of pH values, were found or developed.

The second method for free metal ion determination studied in this thesis was the ion exchange equilibration method. In this method samples are passed through a column containing a small amount of a strongly acidic cation exchange resin until ion exchange equilibrium is achieved between the resin and solution phases. The sorbed metal ions on the resin are then eluted with strong acid and measured quantitatively by atomic absorption or by ICP-AES. A calibration procedure was developed for solution-resin equilibration under non-trace ion exchange conditions and was applied successfully to the determination of free calcium and magnesium in urine samples. With this method, the pH and the ionic strength of the urine sample need not be controlled or adjusted. This makes the procedure very convenient for measurements of these free species in complicated systems such as urine, where pH and ionic strength may vary considerably from sample to sample.

Three kinds of strongly acidic cation exchange resins were investigated for use in this method: Amberlyst 15, Dowex 50W x2 and Dowex 50W x8. Although the exchange characteristics of the Amberlyst 15 resin were reasonable, blocking of the column occurred when urine samples were passed due to particulate matter plugging the small pores of the resin. This led to a study of the other two resins.

A problem was encountered with Dowex 50W x2 in that the resin tended to swell or shrink as a function of the ionic strength of the sample solution being passed through it. This significantly affected the selectivity properties of the resin. The effect of ionic strength could be corrected for by including a term incorporating this factor into the calculation procedure, but it was inconvenient to do.

Investigation of Dowex 50W x8, which had been used previously in ion-exchange equilibration systems using AAS for detection, showed it to be the best of the resins studied because no blocking was observed when urine samples were analyzed. Furthermore, ionic strength over the range seen in normal urine samples did not significantly affect resin selectivity .

Direct pumping of eluent from the resin column into the nebulizer of an ICP-AES instrument was also tested. This approach made the measurement of total sodium, potassium, calcium, magnesium, phosphorus and sulfur in urine simpler and more straightforward. The major problem was the longer integration times (of the order of 100 s) needed for measurement owing to band broadening from the connecting tubing between the column and nebulizer of the ICP, and from the nebulizer itself. These longer integration allowed instrument drift to degrade the precision of the measurement. Also, the ICP torch had to be run continuously for long periods of time, which is expensive.

Compared with other methods for free metal ion determination, the ion-exchange/ICP-AES method has several advantages. (1) The original equilibria of the sample, including pH and ionic strength, are not disturbed. Since no pretreatment of the samples is required before measurement and since samples are pumped through the column until complete breakthrough of the metal ions has occurred, the original sample conditions are not changed by the measurement process. This is a particularly useful feature for kinetically labile systems. (2) Many metal ions can be determined simultaneously with high specificity if an ICP-AES is used as the detector. This makes the method flexible and widely applicable to a variety of metal ion species. (3) By increasing the amount of the resin, very low concentrations of free metal ions can be determined. (4) High concentrations of neutral or negatively charged compounds, either organic or inorganic, do not interfere.

There are also certain restrictions with the method. (1) It must be assumed that only the free, hydrated, metal ion is exchanged onto the resin phase [127] and not, for example, positively charged complexes of a metal with a neutral ligand. This is not a problem when the amount of neutral ligand present in the sample is small, but could be with high concentrations of some ligands. If, however, the ligand is negatively charged, the charge on the metal-ligand complex will be smaller than for the free metal ion. Under these conditions the selectivity of the cation exchange resin for the less positively charged species is usually much lower than for the higher charged species. (2) Although the calibration procedure described in this thesis is particularly useful for samples such as urine, whose composition normally falls within a fairly well defined range, it cannot be used directly on samples whose ionic strength and composition is completely unknown. In order to apply the method it is necessary to prepare an appropriate set of standards, and different kinds of samples require sets of standards that bracket the sample range. (3) Ion-exchange equilibration methods require time for sample equilibration and subsequent measurement. This makes it unsuitable for kinetic studies of systems where the free metal ion concentration changes quickly. By reducing the amount of resin in the column, and decreasing the size of the resin particles, it may be possible to decrease the equilibration time to the point where it could be useful for kinetic studies.

8.2 *Future work*

In the area of spectrophotometric methods of free metal ion measurements a lot of work needs to be done in synthesizing new indicators which have better selectivity and sensitivity for a individual metal ions. Ideally, the indicator should also be pH insensitive to make it applicable to samples over a wide range of acidities. The indicator increment method appears to be suitable only for relatively simple systems. Although the method has some academic interest, for practical applications the lack of applicable indicator compounds makes further study of the method not recommended at this time.

Further work could be done, however, on the sample ion increment method by introducing a slightly different view to the method. Originally, only one addition of metal ion to the sample solution was proposed after the indicator solution had been added [105]. The error associated with the resulting value for the free metal ion concentration is partially determined by how closely the amount of metal ion added matches with the amount of metal-indicator complex formed after the addition of the metal ion. The error is minimum if the two concentrations are equal. Because the amount of metal-indicator complex that will form cannot be known before the metal ion is added and the absorbance measured, the authors suggested an iterative approach in which several identical samples are analyzed successively, the value of the previous metal-indicator complex being used to select the amount of metal ion to be added in the next one [105].

A new approach involving multi-addition of the metal ion is suggested. This can be done by either adding a selected amount of metal ion to the same sample several times after the indicator was added, with the absorbance being measured after each addition of indicator and metal ions, or by adding different amounts of the metal ion to several identical samples after each had received the same amount of indicator, and then measuring the absorbance of the solutions. The amount of metal-indicator complex formed would be determined from absorbance measurements on the solutions and plotted against the total metal ion added to the solution. At first the amount of metal-indicator complex would be larger than the metal ion added, but as more metal ion was added the amount would become larger than the amount of metal-indicator complex formed. The point where the two terms are identical could be determined from the plot. At this point, the amount of metal ion consumed by the indicator is exactly compensated by the amount of metal ion added. The free metal ion concentration could be determined from the amount of indicator added, the amount of metal-indicator complex formed at that point, along with the conditional stability constant of the metal-indicator complex. With this approach, the measurement error of the sample ion increment method would be minimized.

Several additional experiments could be done to improve and expand the applicability of the ion exchange equilibration method. (1) The properties of different kinds of resins could be studied. Weakly acidic cation exchange resins might be useful for the determination of the free concentrations of mono-valent cations in solution if carried out in combination with pH measurements. That is, the equilibrium between the ion exchange of mono-valent cations with hydrogen ion could be investigated. In addition, anion exchange resins might be useful for free anion measurements in solution. (2) Other detection methods, such as ion chromatography or capillary electrophoresis, might be useful alternatives to reduce the cost of the measurements and the time needed for detection, or to provide specific quantitation of ions not determinable by AA or ICP-AES. (3) Miniaturization of the apparatus is desirable to reduce equilibration and detection times as well as the volume of sample required. This would be especially advantageous for clinical use on blood or serum samples. Overall, the ion-exchange equilibration approach is a very versatile method for free metal ion determinations that seems certain to find more applications in the future.

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Appendix I

BASIC Program for Calculating Free Metal Ion Concentration with the Indicator Increment Method

```
10 REM  INDICATOR INCREMENT METHOD, Cases 1 to 3
   REM Check the DATA statements first

INPUT"Which Case ? ";WC

INPUT"Number of indicator additions for each sample=";NC
INPUT"Number of different samples=";NG
INPUT"Number of repeat absorbance measurements for each addition=";NR
INPUT"Number of Wavelength to be used=";NW

PRINT : LPRINT
PRINT"Input the volumes of indicator additions for one sample!"
  FOR I=1 TO NC
  INPUT"V(I)=";V(I)
  LPRINT"     V(I)=";V(I);
  NEXT I
  LPRINT

DIM A(2,NC*NG,NR)
FOR J=1 TO 2
  FOR I=1 TO NC*NG
    FOR K=1 TO NR
    READ A(J,I,K)
    IF J>1 THEN A(J,I,K)=-A(J,I,K)
    NEXT K
  NEXT I
NEXT J
```

```
REM  DATA  Below  512,616nm ; date :
 REM 512nm first
 DATA : REM (sample 1, addition 1, repeat 1), (sample 1, addition 1, repeat 2), ...
              then addition 2,... then sample 2,...
 REM 616nm last
 DATA : REM same order as for 512nm

 REM  1,2=> 512nm,616nm
 ER(1) = 8765 : ER(2) = -19294
 EMR(1) = 21455 : EMR(2) = -1579

 KMR = 256000! : REM conditional stability constant of the metal-indicator complex

IF WC = 1 THEN KML = 204000
IF WC = 2 THEN KML = 171
IF WC = 3 THEN BML1 = 575 : BML2 = 17400

V0 = 20 : CR0 = .001058

  FOR I=1 TO NW
  INPUT"Which Wavelength(1,2 for 512,616nm)=";W(I)
  NEXT I
 W$(1)="512nm   " : W$(2)="616nm   "

LPRINT : LPRINT
LPRINT"THIS IS CASE  ";WC
LPRINT"Number of indicator addition for each sample is ";NC
LPRINT"Number of absorbance measurement for each addition is ";NR
LPRINT"Number of samples =";NG
LPRINT
LPRINT" Wavelength used :    ";
FOR I=1 TO NW : LPRINT W$(W(I)); : NEXT I
T=0:LPRINT
```

```
LPRINT"No.1   No.2     [Mt]0      pMTo      [FL]0      pFLo      [M]0       pMo"
LPRINT"______________________________________________________________________
_____________________________"

100 T=T+1
SMT=0:SFL=0:SM=0
IF T=NG+1 THEN END

FOR L1= 1 TO NC-1:FOR L2=L1+1 TO NC
FOR R1=1 TO NR: FOR R2=1 TO NR
ER=0:EMR=0:A1=0:A2=0
VR1=V(L1) :VR2=V(L2)
K1=(T-1)*NC+L1:K2=(T-1)*NC+L2
        FOR J=1 TO NW
        ER=ER+ER(W(J)):EMR=EMR+EMR(W(J))
        A1=A1+A(W(J),K1,R1):A2=A2+A(W(J),K2,R2)
        NEXT J

300 REM CACULATION OF [M],[R],AND [MR]
        CMR1=(A1-VR1*CR0/25*ER)/(EMR-ER)
        CMR2=(A2-VR2*CR0/25*ER)/(EMR-ER)
        CR1=VR1*CR0/25-CMR1
        CR2=VR2*CR0/25-CMR2
        CM1=CMR1/CR1/KMR
        CM2=CMR2/CR2/KMR

 IF WC<3 THEN 400
   GOSUB 2000
   GOTO 500

400 REM CACULATION OF A,B,C for FL0 calculation
        B1=-V0/25*KML*CM1:B2=-V0/25*KML*CM2
        A1=V0/25-B1:A2=V0/25-B2
        C1=-25/V0*(CM1+CMR1)*A1:C2=-25/V0*(CM2+CMR2)*A2
```

```
450 REM RESULTS [MT]0,[FL]0,AND [M]0
   MT0=(B2*C1-B1*C2)/(B1*A2-B2*A1)
   FL0=(C2*A1-C1*A2)/(B1*A2-B2*A1)
   A=KML:B=KML*(FL0-MT0)+1:C=-MT0
   X=B^2-4*A*C
   M0=(-B+SQR(X))/2/A

500 REM PRINT THE RESULTS
 LPRINT K1;"   ";K2;"    ";
LPRINT USING"##.###^^^^";MT0;
LPRINT"    ";
LPRINT USING"##.###";-LOG(MT0)/LOG(10);
LPRINT"    ";
LPRINT USING"##.###^^^^";FL0;
LPRINT"    ";
LPRINT USING"##.###";-LOG(FL0)/LOG(10);
LPRINT"    ";
LPRINT USING"##.###^^^^";M0;
LPRINT"    ";
LPRINT USING"##.###";-LOG(M0)/LOG(10)

SMT=SMT+MT0:SFL=SFL+FL0:SM=SM+M0

 NEXT R2:NEXT R1
 NEXT L2:NEXT L1

NNC=NC*(NC-1)/2

AVMT=SMT/NNC/NR^2 : AVFL=SFL/NNC/NR^2 : AVM=SM/NNC/NR^2

LPRINT"--------------------------------------------------------------------------------"
LPRINT "Average:    ";
LPRINT USING"##.###^^^^";AVMT;
LPRINT"    ";
```

```
LPRINT USING"##.###";-LOG(AVMT)/LOG(10);
LPRINT"    ";
LPRINT USING"##.###^^^^";AVFL;
LPRINT"    ";
LPRINT USING"##.###";-LOG(AVFL)/LOG(10);
LPRINT"    ";
LPRINT USING"##.###^^^^";AVM;
LPRINT"    ";
LPRINT USING"##.###";-LOG(AVM)/LOG(10)
LPRINT

GOTO 100
600 END

2000 REM Subroutine for case 3 (ML1, ML2 formed)

REM CACULATION OF A,B,C for FL0 calculation
        A=(CM1-CM2)*BML2*(V0/25)^2
        B=(CM1-CM2)*BML1*(V0/25)
        C=CM1+CMR1-CM2-CMR2

REM RESULTS [MT]0,[FL]0,AND [M]0
        X=B^2-4*A*C : IF X<0 THEN 2100
        FL0=(-B+SQR(X))/(2*A)
        MT0=(CM1+CMR1+CM1*BML2*(V0/25)^2*FL0^2
                +CM1*BML1*V0/25*FL0)*25/V0
        M0=MT0/(1+BML1*FL0+BML2*FL0^2)

        FL0=FL0+BML1*M0*FL0+2*BML2*M0*FL0^2

GOTO 2199

2100 LPRINT"NEGATIVE in SQR(), The results of next line are INVALID !!!"
2199 RETURN
```

Appendix II

BASIC Program for Calculating Species Concentrations at Eqiulibrium

```
REM This program is used to calculate the concentration of each species
REM  at equilibrium
REM x metals y ligands M:L =m:n

NK=33:NM=4:NL=3: REM Number of constants, metals and ligands

REM input log(K(MOH)), Charge on the M, ionic strength, from M1 to M(NM)
REM IF one is negligible give log(K(MOH)) a value of -10
DATA 1.3,2,0,2.58,2,0,-0.5,1,0,-0.2,1,0 : REM for Ca,Mg,K,Na
FOR I=1 TO NM
 FOR J=1 TO 3
 READ MOH(I,J)
 NEXT J
KMOH(I)=10^MOH(I,1)
NEXT I
REM CONVERT TO NEW IONIC STRENGTH LATER

LPRINT"Number of K =";NK
DIM K(NK,8),K1(NK),PAM(NM),KML(NM,NL,NK,5)
REM K(NK,1)=>log K,K(NK,2)=>Charge of ML,K(NK,3)=>Charge of M
REM K(NK,4)=>Ionic strength of the literature K,K(NK,5)=>Which Metal
REM K(NK,6)=>Which Ligand
REM K(NK,7)=>m in MmRn : K(NK,8)=>n in MmRn

60 INPUT"pH of solution=";PH
LPRINT"pH of solution=";PH

INPUT"In M C(Ca),C(Mg),C(K),C(Na),C(L),C(P),C(S)=";CM(1),CM(2),
        CM(3),CM(4),CL(1),CL(2),CL(3)
```

```
LPRINT"In M C(Ca),C(Mg),C(K),C(Na),C(L),C(P),C(S)=";CM(1),CM(2),
        CM(3),CM(4),CL(1),CL(2),CL(3)

ISOS=CM(3)+CM(4):REM for this study, the ionic strength was taken as the sum
of sodium and potassium concentrations.

IF ISOS=PISOS THEN 600
IF CCC>0 THEN 300

FOR I=1 TO NK
 FOR J=1 TO 8
 READ K(I,J)
 NEXT J
  K(I,1)=10^K(I,1):K1(I)=K(I,1)
NEXT I

REM DATA LOG(K), Z(ML), Z(M), IONIC STRENGTH, WHICH M, WHICH
        L, m, n
REM in the order of ML,MmLn,MHL,Mm(HL)n,MH2L .....
REM even if you do not have ML, just ML2 for example, you need to enter ML and
        give it a very small K, log(k)=-10 !!!

DATA  4.68,-1,2,0,1,1,1,1,3.09,0,2,0,1,1,1,1,1.1,1,2,0,1,1,1,1,3.37,-1,2,.1,
        2,1,1,1,1.92,0,2,.1,2,1,1,1,.84,1,2,.1,2,1,1,1,.56,-2,1,.15,3,1,1,1,-.3,
        -1,1,.15,3,1,1,1,.7,-2,1,.1,4,1,1,1,.1,-1,1,.15,4,1,1,1

DATA  6.46,-1,2,0,1,2,1,1,2.68,0,2,0,1,2,1,1,.8,1,2,0,1,2,1,1,4.83,-1,2,0,2,
        2,1,1,2.75,0,2,0,2,2,1,1,1.18,1,2,0,2,2,1,1,.6,-2,1,.15.3,2,1,1,.48,-1,1,
        .15,3,2,1,1,-.2,0,1,.3,3,2,1,1,.75,-2,1,.15,4,2,1,1,.6,-1,1,.2,4,2,1,1,
        .114,0,1,.3,4,2,1,1

DATA  2.33,0,2,0,1,3,1,1,2.23,0,2,0,2,3,1,1,.79,-1,1,0,3,3,1,1,.68,-1,1,0,
        4,3,1,1
```

```
REM logBATA,Z(HnL),Z(L),Ionic strength,0,Which ligand,0,0
DATA 5.859,-2,-3,0.1,0,1,0,0,10.297,-1,-3,0.1,0,1,0,0,14.285,0,-3,0,0,1,0,0
DATA 12.35,-2,-3,0,0,2,0,0,19.549,-1,-3,0,0,2,0,0,21.697,0,-3,0,0,2,0,0
DATA 1.97,-1,-2,0,0,3,0,0

300 REM CONVERT to NEW ionic strength

REM CONVERT K(M(OH)) NOW
FOR I=1 TO NM
ZML=MOH(I,2)-1:ZM=MOH(I,2):ZL=-1
Z=ZML:IS=MOH(I,3):GOSUB 5000:ACML1=AC
Z=ZM:GOSUB 5000:ACM1=AC
Z=ZL:GOSUB 5000:ACL1=AC
IS=ISOS:GOSUB 5000:ACL2=AC
Z=ZM:GOSUB 5000:ACM2=AC
Z=ZML:GOSUB 5000:ACML2=AC
KMOH(I)=KMOH(I)*ACML1*ACM2*ACL2/ACML2/ACM1/ACL1
NEXT I

REM CONVERT K(ML) NOW
FOR I=1 TO NK
IF K(I,5)=0 THEN 420
ZML=K(I,2):ZM=K(I,3)
ZL=(ZML-ZM*K(I,7))/K(I,8)
Z=ZML:IS=K(I,4):GOSUB 5000:ACML1=AC
IS=ISOS:GOSUB 5000:ACML2=AC
Z=ZM:GOSUB 5000:ACM2=AC
IS=K(I,4):GOSUB 5000:ACM1=AC
Z=ZL:GOSUB 5000:ACL1=AC
IS=ISOS:GOSUB 5000:ACL2=AC
K(I,1)=K1(I)*ACML1*ACM2^K(I,7)*ACL2^K(I,8)/ACML2/ACM1^K(I,7)
        /ACL1^K(I,8)
GOTO 500
```

```
REM CONVERT BATA(HnL) now
420 IS=ISOS:Z=K(I,2):GOSUB 5000:ACHL=AC
Z=K(I,3):GOSUB 5000:ACL=AC
  IS=K(I,4):GOSUB 5000:ACLO=AC
  Z=K(I,2):GOSUB 5000:ACHLO=AC
  Z=1: GOSUB 5000:ACHO=AC : REM activity coefficient of H in literature
REM According to reference[138], equilibria involving protons have been expressed
        as concentration constants, while in our experiments the activity of H was
        measured with a pH meter. That is why in the following corrections, the
        activity coefficient of H at the ionic strength in the literature was considered.
K(I,1)=K1(I)*ACL/ACHL*ACHLO/ACLO/ACHO^(K(I,2)-K(I,3))
500 XK=LOG(K(I,1))/LOG(10)
     XK=INT(XK*1000+.5)/1000
     LPRINT"  logK'=";XK;
NEXT I

600 REM ALPHA=>PARTITION FACTOR
H=10^(-PH)
FOR I=1 TO NL
K=0:APL(0)=1:SAPL=1
        FOR J=1 TO NK
        IF K(J,5)<>0 THEN 750
        IF K(J,6)<>I THEN 750
        K=K+1
        KH(K)=K(J,1)
        APL(K)=KH(K)*H^K
        SAPL=SAPL+APL(K)
750     NEXT J
        FOR J=0 TO K
        ALPHAL(I,J)=APL(J)/SAPL
        NEXT J
 KK(I)=K
NEXT I
```

```
FOR I=1 TO NL
FL(I)=CL(I)*.5
NEXT I
CIN=0
FOR I=1 TO NM : ALPHAM(I)=1 : NEXT I

900 REM ITERATION METHOD
FOR I=1 TO NL
      FOR J=0 TO KK(I)
      L(I,J)=FL(I)*ALPHAL(I,J)
      NEXT J
NEXT I
FOR I=1 TO NM
 SAKL(I)=0
910   FOR J=1 TO NL
       K=0
            FOR Q=1 TO NK
            IF K(Q,5)<>I THEN 1090
            IF K(Q,6)<>J THEN 1090
            KML(I,J,K,0)=K(Q,1)
            KML(I,J,K,1)=K(Q,7):KML(I,J,K,2)=K(Q,8)
            KML(I,J,K,4)=K(Q,2):KML(I,J,K,5)=K(Q,3)
            KML(I,J,K,3)=(K(Q,2)-K(Q,7)*K(Q,3))/K(Q,8)-(KML(I,J,0,4)-
                  KML(I,J,0,1)*KML(I,J,0,5))/KML(I,J,0,2)
            K=K+1
1090        NEXT Q
1100  AKL(I,J)=KMOH(I)*10^(-14)/H : REM if do not consider MOH then "0"
            FOR Q=0 TO K-1
            AKL(I,J)=AKL(I,J)+KML(I,J,Q,0)*L(J,KML(I,J,Q,3))^KML(I,J,Q,2)
                  *(CM(I)*ALPHAM(I))^(KML(I,J,Q,1)-1)*KML(I,J,Q,1)
            NEXT Q
      QQ(I,J)=K-1
      SAKL(I)=SAKL(I)+AKL(I,J)
      NEXT J
 PAM(I)=ALPHAM(I)
 ALPHAM(I)=1/(1+SAKL(I))
 IF ABS(ALPHAM(I)-PAM(I))/ALPHAM(I)<.01 THEN 1110
 PRINT ALPHAM(I)
 SAKL(I)=0
 GOTO 910
1110  NEXT I

FOR I=1 TO NL
TFL(I)=FL(I)
        FOR J=1 TO NM
                FOR K=0 TO QQ(J,I)
                TFL(I)=TFL(I)+KML(J,I,K,2)*(CM(J)*ALPHAM(J))^
                        KML(J,I,K,1)*(L(I,KML(J,I,K,3)))^
                        KML(J,I,K,2)*KML(J,I,K,0)
                NEXT K
        NEXT J
PRINT TFL(I),CL(I)
NEXT I



EIF=0
FOR I=1 TO NL
IF (ABS((TFL(I)-CL(I))/CL(I)) > 1E-08) THEN  EIF=1
NEXT I
IF EIF=0 THEN 2090

PRINT
FOR I=1 TO NL
FL(I)=FL(I)*CL(I)/TFL(I)
NEXT I
CIN=CIN+1
GOTO 900
```

```
2090 REM OUTPUT

LPRINT
LPRINT"Number of iteration =";CIN
LPRINT

FOR I=1 TO NM
LPRINT"ALPHAM(";I;")=";ALPHAM(I)
NEXT I
LPRINT

FOR I=1 TO NM
LPRINT"M(";I;")=";CM(I)*ALPHAM(I)
        FOR J=1 TO NL
        LPRINT"  M  ";I;"  L  ";J
                FOR K=0 TO QQ(I,J)
                LPRINT"    ";(CM(I)*ALPHAM(I))^KML(I,J,K,1)*
                L(J,KML(I,J,K,3)) ^KML(I,J,K,2)*KML(I,J,K,0);
                NEXT K
        LPRINT
         NEXT J
LPRINT
NEXT I

LPRINT" L   HL   H2L   H3L ... "

FOR I=1 TO NL
        FOR J=0 TO KK(I)
        LPRINT"  ";FL(I)*ALPHAL(I,J);
        NEXT J
LPRINT
LPRINT
NEXT I
```

```
LPRINT" MOH ..."
FOR I=1 TO NM
LPRINT KMOH(I)*CM(I)*ALPHAM(I)*10^(-14)/H;"      ";
NEXT I
LPRINT
CCC=CCC+1
PISOS=ISOS
LPRINT:LPRINT
GOTO 60
END

5000 REM SUBROUTINE FOR ACTIVITY COEFFICIENT
AC=10^(-.511*Z^2*(SQR(IS)/(1+SQR(IS))-.3*IS))
RETURN
```

Appendix III

BASIC Program for Calculating Free Ca^{2+} and Free Mg^{2+} Concentrations in Urine Samples

```
REM  Free Ca, Mg Determination in Urine Samples
AWNA=22.99 : AWK=39.098 : AWCA=40.08 : AWMG=24.305

REM INPUT the coefficients of log(K mix) first
DIM CK(4,5) : REM first row Ca/Na (KR,CaR,MgR,1),2nd Ca/K,then
      Mg/Na,Mg/k
FOR I=1 TO 4
 FOR J=1 TO 5
 READ CK(I,J)
 NEXT J
NEXT I

REM  X8 resin DEC/93
REM  CL system  5 factors (Na,K,Ca,Mg,1) NH4+ exist as main interference
DATA  -.00502,.00388,.00466,.01975,2.40356
DATA  -.00861,.00831,-.0229,-.01014,2.04337
DATA  -.00656,.00177,-.000281,.02078,2.10283
DATA  -.01015,.0062,-.02786,-.00911,1.74274
REM Calculate K mix
INPUT"How many solutions = ";N
DIM  KMIX(4,N),MR(4,N),NAS(N),KS(N),NASS(N),KSS(N),IS(N)
REM MR(4,N)=>NaR,KR,CaR,MgR. Input MR,NaSS,KSS in mM
FOR I=1 TO 4
 FOR J=1 TO N
 READ MR(I,J)
 NEXT J
NEXT I
```

```
FOR I=1 TO N
READ NASS(I)
NAS(I)=NASS(I)/1000
NEXT I
FOR I=1 TO N
READ KSS(I)
KS(I)=KSS(I)/1000
NEXT I

REM NAR   all in mM
DATA  21.3,16.4,20,16.3,20.2,16.3,21.3,16.4,20,16.3,20.2,16.3,
      21.3,16.4,20,16.3,20.2,16.3,21.3,16.4,20,16.3,20.2,16.3

REM  KR
DATA   11.9,10.1,11.3,9.5,11,8.9,11.9,10.1,11.3,9.5,11,8.9,
      11.9,10.1,11.3,9.5,11,8.9,11.9,10.1,11.3,9.5,11,8.9

REM  CaR
DATA  3.07,4.61,2.93,4.71,3.09,4.59,3.07,4.61,2.93,4.71,3.09,4.59,
      3.07,4.61,2.93,4.71,3.09,4.59,3.07,4.61,2.93,4.71,3.09,4.59

REM MgR
DATA  1.74,1.37,1.67,1.43,1.72,1.35,1.74,1.37,1.67,1.43,1.72,1.35,
      1.74,1.37,1.67,1.43,1.72,1.35,1.74,1.37,1.67,1.43,1.72,1.35

REM  NaS
DATA  196,172,196,172,196,172,193,171,193,171,193,171,198,171,
      198,171,198,171,198,173,198,173,198,173

REM  KS
DATA   57.7,51.6,57.7,51.6,57.7,51.6,56.4,50.8,56.4,50.8,56.4,50.8,
      58.2,51.6,58.2,51.6,58.2,51.6,58.5,52.2,58.5,52.2,58.5,52.2
```

```
FOR I=1 TO N
IS(I)=NAS(I)+KS(I)
NEXT I

FOR I=1 TO 4
        FOR J=1 TO N
        KMIX(I,J)=MR(1,J)*CK(I,1)+MR(2,J)*CK(I,2)+MR(3,J)*CK(I,3)
                +MR(4,J)*CK(I,4)+CK(I,5)
         KMIX(I,J)=10^KMIX(I,J)
        NEXT J
NEXT I

REM K(conc.) Determination

DIM LGAC1(N),LGAC2(N),AC1(N),AC2(N),CF(N),KCONC(4,N)

REM IS=>ionic strength,LG=>log,AC=>activity coefficient,
        CF=>correction factor

FOR I=1 TO N
LGAC1(I)=-.511*(SQR(IS(I))/(1+SQR(IS(I)))-.3*IS(I))
LGAC2(I)=LGAC1(I)*4
AC1(I)=10^LGAC1(I)
AC2(I)=10^LGAC2(I)
CF(I)=AC1(I)^2/AC2(I)
NEXT I

FOR I=1 TO 4
        FOR J=1 TO N
        KCONC(I,J)=KMIX(I,J)/CF(J)
        NEXT J
NEXT I
```

```
REM FREE Ca,Mg Determination

DIM FCA(2,N),FMG(2,N) : REM 1=>Na, 2=>K

FOR I=1 TO N
FCA(1,I)=INT(MR(3,I)*NASS(I)^2/MR(1,I)^2/KCONC(1,I)*1000+.5)/1000
FCA(2,I)=INT(MR(3,I)*KSS(I)^2/MR(2,I)^2/KCONC(2,I)*1000+.5)/1000
FMG(1,I)=INT(MR(4,I)*NASS(I)^2/MR(1,I)^2/KCONC(3,I)*1000+.5)/1000
FMG(2,I)=INT(MR(4,I)*KSS(I)^2/MR(2,I)^2/KCONC(4,I)*1000+.5)/1000
NEXT I

REM OUTPUT

LPRINT" Free Ca & Mg Concentration in Urine Samples"
LPRINT
LPRINT" Ca (Na)      Ca (K)      Ca  Average(mM  and ppm) "
FOR I=1 TO N
LPRINT
FCA(1,I),FCA(2,I),(FCA(1,I)+FCA(2,I))/2,40.08*(FCA(1,I)+FCA(2,I))/2
NEXT I
LPRINT

LPRINT" Mg (Na)      Mg (K)      Mg  Average(mM  and ppm) "
FOR I=1 TO N
LPRINT
FMG(1,I),FMG(2,I),(FMG(1,I)+FMG(2,I))/2,24.305*(FMG(1,I)+FMG(2,I))/2
NEXT I

END
```

Appendix IV

BASIC Program for Calculating the Coefficient Matrix for a Particular Column--Calibration of the Column

```
REM BASIC program used to calculate the coefficient matrix

INPUT"NOTE : ";NO$
LPRINT"NOTE: ";NO$
LPRINT

REM  MO  DATA FIRST NA/K/CA/MG  (mM)

DATA  60,60,60,60,100,100,100,100,200,200,200,200,280,280,
      280,280,200,100,100,100
DATA  20,40,80,120,20,40,80,120,20,40,80,120,20,40,80,120,80,40,40,40
DATA  .8,1.6,3.2,4.8,1.6,.8,4.8,3.2,3.2,4.8,.8,1.6,4.8,3.2,1.6,
      .8,3.2,1.6,1.6,1.6
DATA  .8,1.6,4,6,4,6,.8,1.6,6,4,1.6,.8,1.6,.8,6,4,4,1.6,1.6,1.6

REM  MR  DATA SECOND  NA/K/CA/MG  Unit: mM in 10ml flask

DATA  13.45,8.62,5.04,3.97,10.39,7.95,10.29,6.14,13.11,
      11.42,14.63,12.06,17.06,18.4,14.6,16.4,12,9,7.84,7.19
DATA  9.73,11.94,13.53,14.86,3.92,6.47,16.24,12.65,2.65,4.37,
      9.88,11.96,2.35,4.43,7.42,12,8.92,6.83,5.68,4.88
DATA  7.73,8.77,5.99,5.23,3.87,1.27,8.97,2.78,2.71,3.21,.75,1.09,
      2.91,2.03,.757,.426,2.14,2.65,2.17,1.61
DATA  3.22,3.32,2.97,2.62,4.12,4.02,.53,.429,2.11,1,.498,.13,.33,
      .12,1.12,.699,.992,.975,.793,.536
```

```
20      INPUT"No . solution =";N
        LPRINT"No . solution =";N

        INPUT"Kinds of k, factors=";NK,NF
         LPRINT"Kinds of k, factors=";NK,NF

30      DIM  MII(N,N),MIO(N,2*N),K(N,NK),C(N,NF),A(NF,NF),
                X(NF,NK),Y(N,NK),AC(NF,N)
        DIM E(N,NK),MO(N,NK),MR(N,NK),KC(2,N,NK),
                MRT(N),MRP(N,NK),ISOS(N)

REM Caculate KC from MO and MR first, KC(1, , )=>µmole/mM mix,
        KC(2, , )=>%/mM mix
AWNA=22.99 : AWK=39.098 : AWCA=40.08 : AWMG=24.305

REM READ MO Unit: mM
        FOR I=1 TO NK
                FOR J=1 TO N
                READ MO(J,I)
                 NEXT J
        NEXT I

REM READ MR Unit: mM (column and loop kept the same)
        FOR I=1 TO NK
                FOR J=1 TO N
                 READ MR(J,I)
                NEXT J
        NEXT I

REM Calculate K(conc.)  Ca/Na,Ca/K,Mg/Na,Mg/K
FOR J=1 TO N
KC(1,J,1)=MR(J,3)*(MO(J,1)^2)/MO(J,3)/(MR(J,1)^2)
KC(1,J,2)=MR(J,3)*(MO(J,2)^2)/MO(J,3)/(MR(J,2)^2)
KC(1,J,3)=MR(J,4)*(MO(J,1)^2)/MO(J,4)/(MR(J,1)^2)
KC(1,J,4)=MR(J,4)*(MO(J,2)^2)/MO(J,4)/(MR(J,2)^2)
LPRINT" K CA/MG = ";MR(J,3)*MO(J,4)/MR(J,4)/MO(J,3);
        "  K  K/NA = ";MR(J,2)*MO(J,1)/MR(J,1)/MO(J,2)
NEXT J
REM convert to KC mM/mM mix later

REM caculate KC %/mM  Total MRT unit μE
FOR J=1 TO N
MRT(J)=MR(J,1)+MR(J,2)+2*MR(J,3)+2*MR(J,4)
MRP(J,1)=MR(J,1)/MRT(J)*100
MRP(J,2)=MR(J,2)/MRT(J)*100
MRP(J,3)=2*MR(J,3)/MRT(J)*100
MRP(J,4)=2*MR(J,4)/MRT(J)*100
LPRINT" Total mM(equivalence) = ";MRT(J)
NEXT J
REM Caculate KC %/mM  Ca/Na,Ca/K,Mg/Na,Mg/K
FOR J=1 TO N
KC(2,J,1)=MRP(J,3)*MO(J,1)^2/MO(J,3)/MRP(J,1)^2
KC(2,J,2)=MRP(J,3)*MO(J,2)^2/MO(J,3)/MRP(J,2)^2
KC(2,J,3)=MRP(J,4)*MO(J,1)^2/MO(J,4)/MRP(J,1)^2
KC(2,J,4)=MRP(J,4)*MO(J,2)^2/MO(J,4)/MRP(J,2)^2
NEXT J

REM convert to KC mix
 FOR J=1 TO N
 IS=(MO(J,1)+MO(J,2))/1000
 ISOS(J)=IS

REM the following three standards contain NH4+ (ionic stength)
IF J=18 THEN ISOS(J)=ISOS(J)+.02
IF J=19 THEN ISOS(J)=ISOS(J)+.05
IF J=20 THEN ISOS(J)=ISOS(J)+.1
IS=ISOS(J)
```

```
LPRINT"ISOS (";J;")= ";IS;"      ";
 Z=1: GOSUB 50000 : AC1=ACT
 Z=2: GOSUB 50000 : AC2=ACT
 CF=AC1^2/AC2

 PRINT"CF (";J;")=";CF
 FOR I=1 TO NK
  KC(1,J,I)=KC(1,J,I)*CF
  KC(2,J,I)=KC(2,J,I)*CF
  NEXT I
 NEXT J

REM convert to log KC values
  FOR I=1 TO NK
        FOR J=1 TO N
               FOR K=1 TO 2
               KC(K,J,I)=INT(LOG(KC(K,J,I))/LOG(10)*10000)/10000
                NEXT K
        NEXT J
  NEXT I

LPRINT"NO.   k Ca/Na   k Ca/K   k Mg/Na   k Mg/K   mix"
FOR J=1 TO N
LPRINT J;"   ";KC(1,J,1);"   ";KC(1,J,2);"   ";KC(1,J,3);"   ";KC(1,J,4)
NEXT J
LPRINT: LPRINT

REM calculate the coefficients from logK and MR(µmole/mM and %/mM)
FOR BK=1 TO 2
IF BK=2 THEN END
REM Print out NOTE
IF BK=1 THEN LPRINT"Calculations based on KC(micromole/mM)"
IF BK=2 THEN LPRINT"Calculations based on KC(%/mM)"
  LPRINT" MR is Na, K, Ca, Mg !"
```

```
FOR I=1 TO NK
      FOR J=1 TO N
      K(J,I)=KC(BK,J,I)
      NEXT J
NEXT I

FOR I=1 TO NF-1
      FOR J=1 TO N
      IF BK=1 THEN C(J,I)=MR(J,I)
      IF BK=2 THEN C(J,I)=MRP(J,I)
      NEXT J
NEXT I
FOR J=1 TO N
C(J,NF)=1
NEXT J


REM FIND A=C^T*C
FOR I=1 TO NF
      FOR J=1 TO NF
       A(I,J)=0
             FOR K=1 TO N
             A(I,J)=A(I,J)+C(K,I)*C(K,J)
             NEXT K
      NEXT J
NEXT I

REM FIND A^-1
FOR I=1 TO NF
      FOR J=1 TO NF
      MII(I,J)=A(I,J)
      NEXT J
NEXT I
```

```
NN=NF
GOSUB 40000
REM LET A(NF,NF)=A(NF,NF)^-1
 FOR I=1 TO NF
     FOR J=1 TO NF
     A(I,J)=MIO(I,J)
     NEXT J
 NEXT I

REM FIND X MATRIX
 FOR I=1 TO NF
     FOR J=1 TO N
     AC(I,J)=0
          FOR K=1 TO NF
          AC(I,J)=AC(I,J)+A(I,K)*C(J,K)
          NEXT K
     NEXT J
 NEXT I
 FOR I=1 TO NF
     FOR J=1 TO NK
     X(I,J)=0
          FOR K=1 TO N
          X(I,J)=X(I,J)+AC(I,K)*K(K,J)
          NEXT K
     NEXT J
 NEXT I

REM OUTPUT X
LPRINT" X  MATRIX"
FOR I=1 TO NF
     FOR J=1 TO NK
     LPRINT"    ";X(I,J);
     NEXT J
 LPRINT
```

```
NEXT I
LPRINT

REM RECONSTRUCTED Y
FOR I=1 TO N
        FOR J=1 TO NK
        Y(I,J)=0
                FOR K=1 TO NF
                Y(I,J)=Y(I,J)+ C(I,K)*X(K,J)
                NEXT K
        NEXT J
NEXT I

REM ERROR E=(Y-K)/K %
LPRINT"ERROR MATRIX   Y, K, E=(Y-K)/K %"
FOR I=1 TO N
        FOR J=1 TO NK
        Y(I,J)=10^Y(I,J) : K(I,J)=10^K(I,J)
         E(I,J)=(Y(I,J)-K(I,J))/K(I,J)*100
        LPRINT"   ";INT(Y(I,J)*100)/100;"     ";INT(K(I,J)*100)/100;
        LPRINT"  ";INT(E(I,J)*100)/100
        NEXT J
 LPRINT
NEXT I
LPRINT
DIM  SK(NK),SK2(NK),SY(NK),SY2(NK),SKY(NK),DKY2(NK),R(NK),NSEE(NK)

FOR J=1 TO NK
SK(J)=0:SK2(J)=0:SY(J)=0:SY2(J)=0:SKY(J)=0:DKY2(J)=0
        FOR I=1 TO N
        SK(J)=SK(J)+K(I,J)
        SK2(J)=SK2(J)+K(I,J)^2
        SY(J)=SY(J)+Y(I,J)
        SY2(J)=SY2(J)+Y(I,J)^2
        SKY(J)=SKY(J)+K(I,J)*Y(I,J)
        DKY2(J)=DKY2(J)+(K(I,J)-Y(I,J))^2
        NEXT I
R(J)=(SKY(J)-SK(J)*SY(J)/N)/SQR((SK2(J)-SK(J)^2/N)*(SY2(J)-SY(J)^2/N))
NSEE(J)=SQR(DKY2(J)/(N-2))
LPRINT"R, STANDARD ERROR OF ESTAMATION =";R(J),NSEE(J)
LPRINT"AVERAGE OF K, RSEE  = ";SK(J)/N,NSEE(J)/SK(J)*N
LPRINT
NEXT J
LPRINT

NEXT BK
END

40000 REM MATRIX INVERSION SUBR.
FOR I=1 TO NN
        FOR J=1 TO NN
        MIO(I,J+NN)=0
        MIO(I,J)=MII(I,J)
        NEXT  J
MIO(I,I+NN)=1
NEXT I
FOR K=1 TO NN
IF K=NN THEN 40424
M=K
 FOR I=K+1 TO NN
 IF ABS(MIO(I,K)) > ABS(MIO(M,K)) THEN M=I
 NEXT I
 IF M=K THEN 40424
 FOR J=K TO 2*NN
 MIO=MIO(K,J)
 MIO(K,J)=MIO(M,J)
 MIO(M,J)=MIO
 NEXT J
```

```
40424  FOR J=K+1 TO 2*NN
       MIO(K,J)=MIO(K,J)/MIO(K,K)
       NEXT J
       IF K=1 THEN 40434
       FOR I=1 TO K-1
              FOR J=K+1 TO 2*NN
              MIO(I,J)=MIO(I,J)-MIO(I,K)*MIO(K,J)
              NEXT J
       NEXT I

      IF K=NN THEN 40441

40434 FOR I=K+1 TO NN
              FOR J=K+1 TO 2*NN
              MIO(I,J)=MIO(I,J)-MIO(I,K)*MIO(K,J)
              NEXT J
       NEXT I
NEXT K

40441 FOR I=1 TO NN
              FOR J=1 TO NN
              MIO(I,J)=MIO(I,J+NN)
              NEXT  J
       NEXT I
40999  RETURN

50000 REM SUBROUTINE FOR ACTIVITY COEFFICIENT
ACT=10^(-.511*Z^2*(SQR(IS)/(1+SQR(IS))-.3*IS))
RETURN
```